Physical map of the chromosome of the phytopathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola*

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***Pseudomonas syringae* pv. *phaseolicola* (*P.s. phaseolicola*) is one of about 45 recognized pathovars within the *P. syringae* group and is the causal agent of halo-blight disease of beans. DNA from this bacterium digested to completion with two different restriction enzymes, *PacI* and *PmeI*, yielded 15 and 16 fragments, respectively. These were separated using PFGE and sized by comparison to known molecular mass markers. The *P.s. phaseolicola* chromosome was determined to be approximately 5.64 Mb in size. To link the different fragments obtained into a circular chromosome map for both enzymes, 150 random Tn5 mutants of *P.s. phaseolicola* were used as a source of DNA and the identification of the band carrying the transposon 'tag' in each mutant was done after PFGE and Southern hybridization of a complete chromosomal digestion using a Tn5 probe. Partial digestions of DNA from different Tn5 mutants 'tagging' specific bands were then generated and the complete and partial products of the digestion separated by PFGE and identified with a Tn5 probe. By calculating the size of the partial products, it was then possible to link different bands into a physical map. This is the first report on the construction of a physical map of a member of the *P. syringae* group and should be invaluable for molecular genetic analysis in this species and in evolutionary or taxonomic studies when compared to similar data obtained for any of the other recognized pathovars.**

Keywords: chromosome size, physical map, *Pseudomonas syringae* pv. *phaseolicola*, pulsed-field gel electrophoresis, transposons

INTRODUCTION

Research on plant-pathogenic bacteria at the molecular level is being carried out in many academic and industrial laboratories throughout the world because of the need for a better understanding of the basic principles that govern bacteria–plant interactions if new and lasting strategies of control are to be found. *Pseudomonas syringae* is a large group of phytopathogenic bacteria comprising 45 different pathovars which cause disease on a wide range of plant hosts (Young *et al.*, 1992). Of these, *P. syringae* pv. *phaseolicola* (*P.s. phaseolicola*) is the causal agent of 'Halo blight' disease of beans (*Phaseolus vulgaris* L.) which affects large extensions of rain-fed fields throughout Mexico and Central and South America.

P.s. phaseolicola produces a toxin known as phaseolotoxin which is synthesized at 16–18 °C (Goss, 1970),

together with a phaseolotoxin-resistant ornithine carbamoyltransferase, the product of the *argK* gene, which confers immunity to this bacterium against its own toxin (Mitchell, 1978; Peet & Panopoulos, 1987) and which has been cloned and characterized (Mosqueda *et al.*, 1990; Hatziloukas & Panopoulos, 1992). In our laboratory we are using *P.s. phaseolicola* as a model system to study the molecular mechanisms involved in the pathogenic interaction with its host plant. We are also interested in studying genome organization and evolution within the *P. syringae* group which still lacks well-developed molecular genetics and, to date, no comprehensive genetic or physical map for any member of this group is available. As a first step toward the analysis of genome organization and as a tool for genetic and evolutionary analysis we decided to construct a physical map of *P.s. phaseolicola*.

The availability of techniques such as PFGE provides the

means to separate and accurately measure the size of large DNA fragments or even whole chromosomes (Schwartz & Cantor, 1984; Smith *et al.*, 1987a; Snell & Wilkins, 1986; Sor, 1988). This technique combined with the use of restriction enzymes that cleave infrequently within the bacterial chromosome has been applied to estimate the sizes of circular chromosomes of several different bacteria (Smith *et al.*, 1987b; Pyle *et al.*, 1988; Lee & Smith, 1988; Kauc *et al.*, 1989; Lee *et al.*, 1989; Manna & Das, 1993, 1994) including those of four different strains of *P. aeruginosa* (Römling *et al.*, 1989; Hector & Johnson, 1990) and *P. fluorescens* (Rainey & Bailey, 1996). We estimated the size of the *P.s. phaseolicola* chromosome to be 5.64 Mb (5.641 ± 0.13) and that it is a single circular chromosome yielding 15 and 16 *PacI* and *PmeI* restriction fragments, respectively.

To establish the relative position of the different fragments obtained with each restriction enzyme, 'tagging' each individual fragment was used as an alternative to double restrictions and two-dimensional PFGE. To this end, total DNA from 150 Tn5 mutants of *P.s. phaseolicola* was obtained and by using *PacI* and *PmeI*, PFGE and Southern blotting with a Tn5-specific probe, it was possible to readily identify the specific *PacI* or *PmeI* band carrying a transposon 'tag' and construct a physical map of the genome. Also, we have found some mutants in which promoter activity is dependent on temperature or the presence of plant extracts. The corresponding mutations together with the *argK* gene and five transcriptional units for rRNA have been located on the physical map obtained in this work.

METHODS

Bacterial strain and culture conditions. *P.s. phaseolicola* strain Riverside was kindly provided by Dr Noel T. Keen (University of California, Riverside) and was routinely grown in Kings medium B (KB; Lelliott & Stead, 1987) at 28 °C.

Preparation of chromosomal DNA. Chromosomal DNA of *P.s. phaseolicola* was prepared according to Smith *et al.* (1988) with some modifications. The cells were grown at 28 °C overnight on a rotary shaker in 5 ml KB. From this culture 1 ml was inoculated into 50 ml fresh KB in a 250 ml Erlenmeyer flask. The culture was grown at 28 °C and shaken (250 r.p.m.) until the density of the culture reached 70 Klett units (using a red filter). The cells were chilled to 4 °C, harvested by centrifugation at 5000 g for 15 min and washed in 50 ml Pett IV buffer (0.01 M Tris/HCl, pH 7.6, 1 M NaCl) followed by centrifugation at 5000 g for 10 min. The cells were finally resuspended in 8 ml Pett IV at 4 °C. The suspension was warmed to room temperature for 15 min and mixed with an equal volume of 1.2% (w/v) low-melting-temperature agarose (Sea Plaque GTG agarose; FMC) made up in sterile water at 42 °C. The resulting suspension was poured into a mould chamber (Bio-Rad). Solidified blocks were immersed in EC lysis solution (6 mM Tris/HCl, pH 7.6, 1 M NaCl, 100 mM EDTA, pH 7.6, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 1 mg lysozyme ml⁻¹, 10 mg DNase-free RNase ml⁻¹) and incubated at 37 °C for 18 h. The solution was changed to ESP (0.5 M EDTA, pH 8.0, 1% sodium lauryl sarcosine, 1 mg proteinase K ml⁻¹) and

incubated for 48 h at 50 °C. Proteinase activity was inhibited by washing the blocks three times for 1 h at 50 °C in 40 mg PMSF ml⁻¹. The blocks were then stored in 0.05 M EDTA, pH 8.0, at 4 °C.

Digestion of chromosomal DNA with restriction endonucleases. Agarose blocks containing *P.s. phaseolicola* chromosomal DNA were subjected to single digestions with restriction endonucleases. Each agarose block was washed three times in 50 vols TE buffer (10 mM Tris/HCl, pH 7.6, 1 mM EDTA) prior to digestion and then equilibrated with the corresponding digestion buffer at 4 °C for 30 min. DNA was digested with *PacI* or *PmeI* at 37 °C for 16–20 h in 0.25 ml of the restriction buffer recommended by the manufacturer. For total digestion of DNA, 20 U restriction endonuclease was used for each agarose block. When using *I-CeuI*, 1.3 U of enzyme was used, incubating the blocks at 37 °C for 3 h. The digestions were terminated by the addition of 100 mM EDTA, pH 8.0. To partially digest DNA with *PacI* or *PmeI*, the reactions were performed as described above but allowing the digestion to proceed for only 5 h. Alternatively, 4 U restriction endonuclease was used for each agarose block at 37 °C for 16–20 h.

PFGE. PFGE was performed in 1.2% agarose (electrophoresis grade, Ultra Pure, Gibco-BRL) in 0.5× TBE buffer (0.5× TBE buffer is 0.045 M Tris/borate, 1 mM EDTA, pH 8.0). The electrophoresis was regularly run at 14 °C in a CHEF DR III apparatus from Bio-Rad using a linear increase in the pulse intervals. To resolve the shorter fragments pulse times were ramped from 8 to 24 s for 48 h at 165 V. For separation of fragment sizes between 150 and 550 kb the gel was run for 48 h at 150 V with a pulse time from 15 to 60 s. High molecular mass fragments (>600 kb) were resolved using 60–120 s ramped pulse times for 48 h at 150 V. A λ DNA ladder (monomer 48.5 kb) from New England Biolabs was used for size marking. However, DNA fragments greater than 582 kb were resolved and sized by comparison with the *Saccharomyces cerevisiae* YPH80 chromosomes from Bio-Rad (size range 225–1900 kb). Following electrophoresis, gels were stained with ethidium bromide (1 mg ml⁻¹) for 30 min, then destained in distilled water. The DNA bands were visualized with a UV transilluminator.

PFGE of large fragments generated with *I-CeuI* was carried out under different conditions to resolve the small or large fragments. For the three smaller fragments PFGE was performed in 1.2% agarose in 0.5× TBE buffer using 60–120 s ramped pulse times for 66 h at 150 V. Resolution of the five fragments obtained, including the largest two, was achieved by using 0.8% agarose in 0.5× TBE, 500 s pulse, and allowing the separation to proceed for 68 h at 150 V. The smaller fragments were sized against the λ DNA ladder and the *S. cerevisiae* YPH80 chromosomes. The larger fragments were sized against the chromosomes of the yeast *Hansenula wingei* (1.03–3.30 Mb range) purchased from BRL (Mega Base IV DNA Standard).

Southern blots and probe labelling. From a previously obtained collection of Tn5 mutants of *P.s. phaseolicola* (R. Marsch-Moreno, G. Hernández-Guzmán & A. Alvarez-Morales, unpublished), 150 strains were chosen at random and used to prepare and digest their chromosomal DNA as mentioned above. After PFGE, the DNA was depurinated and transferred to a Hybond-N membrane (Amersham) following standard techniques (Sambrook *et al.*, 1989). Plasmid pUI244, a pBR322 derivative containing Tn5 (R. Marsch-Moreno and others, unpublished), was radioactively labelled with [³²P]dCTP (New England Nuclear) by random priming using

commercial kits (Gibco-BRL; Random Primers DNA Labeling System). Hybridization was done at 65 °C overnight, washed once with 0.1% SDS, 0.05% SSC (SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) at room temperature and twice with 0.1% SDS and 0.1% SSC at 65 °C.

PCR-derived probes. Tn5 insertions could not be found in fragments A–E of the *PmeI* restriction products. Therefore, to generate A-specific probes to identify partial digestion products, the A band was isolated from a preparative gel of chromosomal DNA from wild-type *P.s. phaseolicola* and used as template in a PCR reaction. These gels, from which the low molecular mass bands were going to be isolated, also allowed us to confirm that there were no smaller bands than those already identified in the PFGE gels. The agarose pieces containing the appropriate band to be used as template were melted in a boiling-water bath and diluted with an equal amount of distilled water. Three tenfold dilutions were obtained from this solution and stored at 4 °C. PCR reactions were performed in an overall reaction volume of 50 µl using 10 µl of the 10⁻² and 10⁻³ dilutions of template DNA, 5 µl of a random 10 base oligonucleotide as primer and 35 µl of a commercially premixed PCR solution (Gibco-BRL; PCR SuperMix). The PCR reaction was performed using an initial cycle of 94 °C for 2 min, then 40 cycles of 94 °C for 1 min, 37 °C for 2 min and 72 °C for 2 min, followed by a final cycle of 72 °C for 7 min. When specific fragments were amplified, these were eluted from an agarose gel, labelled with ³²P and used as probes to specifically identify complete and partial *PmeI* digestion products of *P.s. phaseolicola*.

RESULTS

Selection of suitable enzymes for PFGE analysis of *P.s. phaseolicola*

Long-range restriction mapping of bacterial genomes requires restriction endonucleases that cut only rarely on the chromosome. Criteria for the selection of appropriate enzymes are G + C content, oligonucleotide frequency and the degree of methylation of the organism to be mapped. *Pseudomonas* has a G + C content of 67% and the tetranucleotide CTAG occurs more than an order of magnitude less frequently than calculated from mononucleotide frequencies (McClelland *et al.*, 1987). Therefore, any restriction enzyme containing only A, T or A, T plus CTAG in its recognition sequence is a promising candidate for an appropriate rare-cutter. Several restriction endonucleases were chosen to be tested for the digestion of total DNA from *P.s. phaseolicola*. Of these, the enzymes *PacI* (5' TTAATTAA) and *PmeI* (5' GTTTAAAC) contain only or predominantly A + T base pairs in their recognition sequences, and *SpeI* (5' ACTAGT) and *XbaI* (5' TCTAGA) contain the tetranucleotide CTAG within their recognition sequences. *XbaI* generated more than 40 bands of less than 50 kb and few fragments of around 150–250 kb. Treatment with *SpeI* generated around 30 fragments, few of them ranging between 150–250 kb but the majority of the fragments being smaller (data not shown). *PacI* and *PmeI* digestion produced 15 and 16 fragments, respectively, ranging in size from 25 to 900 kb. These fragments were efficiently separated by one-dimensional PFGE using a linear increase in the pulsed intervals with the appropriate conditions for resolving each restriction

fragment. When undigested *P.s. phaseolicola* DNA was subjected to PFGE at short and long pulse times, three bands were usually clearly resolved in these gels. These bands did not correspond to any of the *PacI*- or *PmeI*-generated fragments which strongly suggests that such bands are plasmids present in our particular strain (data not shown).

Size of the *P.s. phaseolicola* chromosome

The molecular size of PFGE-separated fragments for both *PacI* and *PmeI* was determined by obtaining computer images of ethidium bromide-stained PFGE gels and using appropriate software to measure distances of band migration compared with both a λ DNA ladder and *S. cerevisiae* YPH80 chromosome markers (Fig. 1). The data were analysed by linear regression. The size of the chromosome was determined by taking the sum of the sizes of individual restriction fragments obtained with *PacI* and *PmeI* enzymes from at least three independent gels. The chromosome size was determined to be 5.641 ± 0.13 Mb or 5.64 Mb (Table 1).

Southern hybridization analysis of a collection of Tn5 mutants after complete and partial digestion of genomic DNA with *PacI* and *PmeI*

Total DNA from each of the Tn5 insertion mutants was subjected to complete digestion with both *PacI* and *PmeI*. After PFGE the DNA was transferred to a membrane and probed with a radioactively labelled plasmid containing Tn5. It was then possible to identify within the bands of the *PacI* or *PmeI* patterns where the transposon had inserted. Furthermore, hybridization also indicated the corresponding bands of both digestion patterns (Fig. 2a). As expected, Tn5 insertions were found more often in the larger fragments and few or none in the smaller ones. A list of representative Tn5 insertions is shown in Table 2.

The use of non-specific primers and PCR to generate hybridization probes for large restriction fragments has been reported (Poddar & McClelland, 1992). Therefore, in those instances where a Tn5 tag could not be found for some of the bands obtained with either restriction enzyme, specific probes were obtained by PCR using DNA isolated from a preparative gel and random primers (10 bases) as a template or by directly using the gel-isolated DNA as probe after labelling with ³²P. This information allowed us to determine the relative order of each fragment within the circular chromosome of *P.s. phaseolicola*. Such was the case for bands A–E of the *PmeI* digestion pattern. Therefore, bands B and E were isolated from a preparative gel, labelled with ³²P and used to probe a filter containing *PacI* and *PmeI* digestions of wild-type *P.s. phaseolicola*. As expected, this probe specifically identified band E of the *PmeI* digestion, but it also specifically identified bands 8 and 9 of the *PacI* pattern, showing that these two bands were linked. This method allowed the unambiguous identification of bands in a complete digestion. However, due to small

Fig. 1. Restriction patterns obtained with the endonucleases *PacI* and *PmeI*. (a) Lanes: 1, λ DNA ladder (the lowest band corresponds to the 97 kb band); 2, resolution of bands C-H of the complete *PmeI* digestion. (b) Lanes: 1, complete *PmeI* digestion; 2, *S. cerevisiae* YPH80 marker starting at 225 kb; 3, λ DNA ladder starting from 48.5 kb; 4, complete *PacI* digestion. (c) Schematic representation of the *PmeI* and *PacI* digestion patterns shown in (b) and band designation.

amounts of chromosome contamination which co-migrate with these bands, it was not always possible to use them to identify partial digestion products.

As for band A, a PCR-derived probe was obtained using DNA from this band as template. At least 40 different random 10-mers were used as primers and one yielded a specific amplification product. This amplified band was used as a probe and shown to be completely specific for band A of the *PmeI* digestion pattern. When using this material to probe a *PacI* total digestion, band 7 was unequivocally identified (data not shown). The fact that it was possible to accurately identify one specific band allowed for the precise mapping of bands linked to the tagged fragment through partially digested DNA. It was then possible to identify from one to five partial products

depending on the size of the specific fragment being tagged (Fig. 2b).

Circular linkage map of the *P.s. phaseolicola* chromosome

DNA from different Tn5 mutants was subjected to partial restriction and the different products identified by Southern blot hybridization with the Tn5-specific probe. This allowed us to identify the specific partial products for different bands of the *PacI* or *PmeI* patterns. To obtain accurate measurements of partially digested DNA from the Tn5 mutants, at least two different PFGE conditions were always used to clearly resolve large and small fragments. Furthermore, in those cases where

Table 1. Size of fragments obtained after complete digestion of the *P.s. phaseolicola* chromosome with *PacI* and *PmeI*

Fragment sizes are the means of at least three different experiments.

<i>PacI</i>		<i>PmeI</i>	
Fragment	Size (kb $\pm$ SD)	Fragment	Size (kb $\pm$ SD)
1	27 $\pm$ 1	A	29 $\pm$ 4
2	62 $\pm$ 1	B	42 $\pm$ 2
3	109 $\pm$ 1	C	141 $\pm$ 4
4	132 $\pm$ 2	D	164 $\pm$ 6
5	152 $\pm$ 2	E	171 $\pm$ 5
6	166 $\pm$ 1	F	178 $\pm$ 3
7	262 $\pm$ 2	G	194 $\pm$ 3
8	276 $\pm$ 2	H	209 $\pm$ 6
9	337 $\pm$ 2	I	270 $\pm$ 20
10	397 $\pm$ 6	J	323 $\pm$ 17
11	573 $\pm$ 26	K	463 $\pm$ 15
12	702 $\pm$ 19	L	488 $\pm$ 13
13	726 $\pm$ 17	M	655 $\pm$ 14
14	843 $\pm$ 2	N	721 $\pm$ 18
15	876 $\pm$ 4	O	755 $\pm$ 19
		P	839 $\pm$ 20
Sum	5640	Sum	5642
Chromosome size: 5641 kb			

more than one Tn5 strain tagging the same restriction fragment were available, DNA samples from at least two independent mutants were used to confirm hybridization data.

Initially the linkage for the different fragments generated with *PacI* was obtained using data from the partial digestions and a preliminary circular map was deduced. This preliminary map was confronted with the cross-hybridization data for the *PacI* and *PmeI* bands and some minor changes were introduced for map areas where previous data had been ambiguous. A final version was obtained using specific partial digestion data for *PmeI* to confirm specific linkages. The data obtained are summarized in Fig. 3.

Locating rRNA operons, promoter-probe-derived mutants and the *argK* gene of *P.s. phaseolicola* within the physical map

A 1.5 kb DNA fragment corresponding to the *Xanthomonas campestris* pv. *campestris* *rrs* (16S) structural gene was labelled with 32 P and used as probe to localize the corresponding gene of *P.s. phaseolicola*. Hybridization signals were obtained in bands 10, 11, 13, 14 and P, N, O, M of the *PacI* and *PmeI* digestions, respectively, and thus, at least four *rrs* genes could be unambiguously identified (Fig. 4a). However, the intensity of the signal

Fig. 2. Identification of bands carrying a Tn5 insertion (tag) and identification of specific partial restriction products. (a) DNA from strains 125, 120, 115 and 90 was restricted with both *PmeI* (○) and *PacI* (◆), the digestion products transferred to a membrane and hybridized to a Tn5 probe. The Tn5 tag in mutants 125 and 115 was located in bands M and 14 of the *PmeI* and *PacI* patterns, respectively. As for mutants 120 and 90, the tag was in bands N and 11 of the *PmeI* and *PacI* patterns, respectively. Some partial products can be identified. (b) DNA from mutants having tags in *PacI* bands 8, 2, 12, 10, 13 and 9. DNA was transferred to a membrane and probed with Tn5 DNA. The arrowheads indicate those partial products that could be identified and sized to provide linkage data. The total digestion product is the stronger lowest band in each lane and corresponds to the tagged band.

corresponding to bands 14 and M was stronger than for the other bands, suggesting that within the map region spanned by these bands there could be more than one region corresponding to another *rrs* gene. To determine unambiguously the number of ribosomal genes, I-*CeuI*, an intron-encoded endonuclease which cleaves specifically within the bacterial *rrl* (23S) genes (Liu *et al.*, 1993), was used to digest total DNA from *P.s. phaseolicola*.

Five distinct bands were obtained with I-*CeuI*. When sizing these DNA fragments it was noticeable that under the PFGE conditions used, their sizes were a mean of 8% different from those calculated previously for similar-sized *PacI* or *PmeI* fragments. This error was much more evident for the two larger I-*CeuI* bands when sized against the *H. wingei* markers. However, anomalous mobilities have been reported for the *H. wingei* markers under certain PFGE conditions (Jones *et al.*, 1989). Based on the fact that four of the *rrs* genes could be clearly assigned to specific *PacI* and *PmeI* fragments by hybridization, and using the 8% error margin, it was possible to calculate accurately the size of the I-*CeuI*

Table 2. Localization of Tn5 in the bands generated by restriction with *PacI* and *PmeI* of total DNA from some *P.s. phaseolicola* mutants

Strain designation	Tn5 localization	
	<i>PacI</i>	<i>PmeI</i>
UIC-1	6	H
UIC-2	9	G
UIC-5	13	O
UIC-10	15	K
UIC-18	10	P
UIC-34	8	I
UIC-35	6	P
UIC-45	5	L
UIC-66	12	P
UIC-75	12	L
UIC-80	3	O
UIC-85	2	H
UIC-90	11	N
UIC-115	14	M
UIC-145	15	J
UIC-150	13	F

fragments which turned out to be 0.196, 0.471, 0.912, 1.492 and 2.616 Mb, a total of 5.68 Mb compared to the 5.64 Mb estimated with the *PacI* and *PmeI* values (Fig. 4b). The *rrs* hybridization data together with the I-CeuI results unambiguously revealed the presence of five sets of ribosomal genes located on the chromosome of *P.s. phaseolicola* as shown in Fig. 5.

To identify genes whose expression is temperature-dependent and which may be linked to phaseolotoxin synthesis, we constructed a promoter-probe derived

from Tn5 which carries a promoterless chloramphenicol acetyltransferase (*cat*) gene, allowing for positive selection of promoters after transposition (R. Marsch-Moreno, G. Hernández-Guzmán & A. Alvarez-Morales, unpublished). With this element we have mutagenized *P.s. phaseolicola*, selecting for Cm^R at 18 °C, and have found several mutants with this response pattern. Also, we have found some mutants in which promoter activity seems to be dependent on the presence of plant extracts. The characteristics of these mutants are summarized in Table 5 and their location, together with that of the *argK* gene are shown in Fig. 5.

DISCUSSION

A physical map of the *P.s. phaseolicola* genome has been constructed by ordering the 15 and 16 large restriction fragments generated with the enzymes *PacI* and *PmeI*, respectively. Most of the restriction fragments were tagged with Tn5 insertions and the map was generated after Southern blot analysis of the fragments obtained by complete and partial restriction digests. PCR-derived probes and bands isolated from agarose gels from fragments which were not tagged were used as probes. The values reported for the chromosome size of four different *P. aeruginosa* strains ranged between 5.2 and 7.0 Mb (Römling *et al.*, 1989; Hector & Johnson, 1990; Schmidt *et al.*, 1996). We have determined the size of the single circular chromosome of *P.s. phaseolicola* to be 5.64 Mb, a value similar to those reported for some *P. aeruginosa* strains, but smaller than the 6.63 Mb chromosome of *P. fluorescens* (Rainey & Bailey, 1996).

When the different fragments obtained with both *PacI* and *PmeI* were linked, a circular map could be readily obtained. The use of an already available collection of Tn5 insertion mutants proved extremely useful since it allowed us to identify strains where the Tn5 insertion was tagging a specific band. Partial digestion of DNA

Fig. 3. Fragment linkages through partial restriction and cross-hybridization data. The arrows indicate those fragments linked through partial restriction data; the black dot indicates the Tn5-tagged band used in the analysis. The hatched bars indicate cross-hybridization obtained using different types of probes: vertical hatching, PCR-derived probe from band A; horizontal hatching, band B and E used as probes after gel elution; diagonal hatching, Tn5 probe. Fragment sizes are not drawn to scale.

Table 3. Location and relevant phenotype of promoter-probe-derived mutants and the *argK* gene of *P.s. phaseolicola*

Allele	Location		Phenotype/selection
	<i>PacI</i>	<i>PmeI</i>	
UIM-21	13	O	Cm ^R at 16 °C in KB medium
UIM-B	13	O	Cm ^R at 16 °C in M9 medium
UIM-C	8	I	Cm ^R at 16 °C in M9 medium
UIM-E	12	L	Cm ^R at 16 °C in M9 medium
UIM-F	6	H	Cm ^R at 16 °C in M9 medium
UIM-E24	14	M	Cm ^R /plant extract/28 °C/KB medium
UIM-E4	14	M	Cm ^R /plant extract/28 °C/KB medium
<i>argK</i>	8	I	Phaseolotoxin-insensitive OCTase

Fig. 4. Determination of number and position of putative rRNA transcriptional units of *P.s. phaseolicola*. (a) *PacI*- and *PmeI*-digested DNA hybridized with a 1.5 kb DNA fragment corresponding to the *X. campestris* pv. *campestris* *rrs* (16S) structural gene. The bands showing a hybridization signal are labelled for each restriction pattern. Both lanes in each digest correspond to slightly different amounts of the same DNA. (b) Lane *Ceu* shows the five bands obtained after digestion of chromosomal DNA from *P.s. phaseolicola* with I-*CeuI* (arrows). Lane *Hw* corresponds to the *H. wingei* DNA marker. **Fig. 5.** Physical map of *P.s. phaseolicola*. The map is drawn to scale. The location of five putative *rrn* transcriptional units, *argK* and some promoter-probe-derived mutants is indicated.

from these mutants yielded up to five different and readily identifiable partial restriction fragments using a Tn5 probe. This information, together with the data provided by linking *PacI* and *PmeI* bands through the Tn5 tag made the use of two-dimensional PFGE analysis unnecessary.

The analysis of 150 independently obtained Tn5 mutants did not allow us to identify Tn5 insertions in each of the *PacI* or *PmeI* restriction products. Therefore, when it became necessary to generate probes for these fragments, the easiest way to do it was to isolate the particular band from a preparative gel and label it with ³²P through random priming. This yielded probes that were very often contaminated with small amounts of co-migrating chromosomal DNA but were nevertheless good enough to identify complete digestion products,

and in some cases, where background due to the contaminant DNA was not high, even partial digestion products could be unambiguously identified. Another way to obtain specific probes is through PCR, which yields completely specific probes, but only if a large collection of random primers is available and only after trying several of these which could prove to be expensive and time-consuming. A similar method based on the use of Tn917 insertions has been used to assign restriction fragments from different strains of *Bacillus subtilis*, known to carry an insertion in a genetically defined gene, to a well-developed genetic map, thus obtaining a correlated physical and genetic map (Ventura & Weiss, 1989).

Since most or all of the genes involved in the synthesis of phaseolotoxin, including *argK*, seem to be linked (Zhang *et al.*, 1993), it became interesting to map the *argK* gene and the different alleles already identified through the use of a promoter-probe transposon. As shown in Table 3 and Fig. 5, *argK* overlaps fragments 8 and I on the physical map and another mutant, UIM-C, selected for its ability to express Cm^R at 16 °C, falls within this same segment. Since phaseolotoxin synthesis occurs at 16 °C, it would not be surprising to find that mutant UIM-C is involved in this phenomenon. We also found that some mutants, such as UIM-E24 and UIM-04, could express Cm^R only in the presence of plant extracts. These mutants would seem good candidates to carry the Tn5 insertion on genes involved in pathogenicity and/or virulence and they map in a completely different position within the physical map, suggesting that not all the genes, operons or regulons involved in the pathogenic process are clustered. The other group of mutants mapped and which also respond to temperature may be linked to the infection process but this remains to be tested.

In *P. aeruginosa* and *P. fluorescens* the rRNA genes are organized as transcriptional units with the 5'-*rrs-rrl-rrf*-3' structure typical of eubacteria (Hartmann *et al.*, 1986; Rainey & Bailey, 1996). To locate the ribosomal genes of *P.s. phaseolicola*, two approaches were taken: hybridization with an *rrs* probe to locate genes encoding 16S RNA and restriction with *I-CeuI* which cleaves within the *rrl* gene encoding 23S RNA. The data obtained in both cases identified five sites on the chromosome where these genes were located, indicating a close linkage between the *rrs* and the *rrl* genes. Therefore, it would seem reasonable to expect that the genes encoding rRNA that have been identified in this work are part of a typical 5'-*rrs-rrl-rrf*-3' transcriptional unit similar to those identified in *P. aeruginosa* and *P. fluorescens*, and maintained in the closely related *P. syringae*.

Furthermore, it is interesting to note that for *P. aeruginosa* PAO, four rRNA operons have been identified (Römling *et al.*, 1989; Holloway *et al.*, 1994), whereas for *P. fluorescens* five such structures were found (Rainey & Bailey, 1996). In the case of *P.s. phaseolicola* five putative *rrn* transcriptional units have been unambiguously identified in this study. Their

position on the circular chromosome is shown in Fig. 5 (labelled *rrnA-rrnE*) where names have been assigned to follow the pattern that most closely resembles that of *P. fluorescens* to demonstrate possible similarities, such as the pair-wise arrangement of the structures, *rrnA-rrnB* and *rrnD-rrnE*, and the clustering in one half of the chromosome with a separation of less than 1.0 Mb, except for *rrnC* (Rainey & Bailey, 1996). These similarities may be expected due to the close relatedness of *P.s. phaseolicola* and *P. fluorescens*, both being bacteria that interact with plants, as a pathogen and as a saprophyte, respectively. These similarities may therefore suggest that the putative *rrn* transcriptional units of *P.s. phaseolicola* may also be atypical of Gram-negative bacteria, being organized as inverted repeats and having a direction of transcription opposite to the direction of DNA replication. Likewise, the origin of replication of the chromosome may be expected to be found between *rrnA* and *rrnE* (Rainey & Bailey, 1996; Brewer, 1988). However, all these speculations remain to be tested and further work is being undertaken in our laboratory to address these issues.

The physical map generated for this bacterium will serve as the framework on which to build a combined physical and genetic map. Work in this laboratory is under way to determine the direction of transcription of the five rRNA operons, to locate the origin of replication of the chromosome and to map genes involved in pathogenicity, toxin production, hyper-sensitive response and avirulence, among others. The information derived from such a map should prove useful in understanding the evolution of this very important group of plant-pathogenic bacteria and in this context it should be interesting to compare some features of the physical/genetic map of *P.s. phaseolicola* to the other 45 pathovars of the *P. syringae* group and to the closely related *P. fluorescens*.

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