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Avirulence gene D of *Pseudomonas syringae* pv. *tomato* may have undergone horizontal gene transfer

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Abstract Avirulence gene D (*avrD*) is carried on the B-plasmid of the plant pathogen *Pseudomonas syringae* pv. *tomato* with plasmid-borne *avrD* homologs widely distributed among the Pseudomonads. We now report sequences in the soft rot pathogen *Erwinia carotovora* that cross-hybridize to *avrD* suggesting a conserved function beyond avirulence. Alternatively, *avrD* may have been transferred horizontally among species: (i) DNA linked to *avrD* shows evidence of class II transpositions and contains a novel IS*3*-related insertion sequence, and (ii) short sequences linked to *avrD* are similar to pathogenicity genes from a variety of unrelated pathogens. We have also identified the gene cluster that controls B-plasmid stability.

Key words: Insertion sequence; IS*1240*; *avrD*; *Pseudomonas syringae* pv. *tomato*

1. Introduction

The plant pathogen, *Pseudomonas syringae* pv. *tomato* (*Pst*), causes bacterial speck disease on tomato. The PT23 strain of *Pst* carries four plasmids designated A, B, C and D [1]. The B-plasmid of *Pst* is about 83 kbp in size and contains avirulence gene *D* (*avrD*) which is defined by its ability to confer avirulence on the related pathogen *Pseudomonas syringae* pv. *glycinea* (*Psg*) in its interaction with soybean [2–4]. The *avrD* gene has been mapped to a 5.6 kbp *Hin*dIII fragment. DNA sequencing revealed a cluster of at least five open reading frames (ORFs) arranged in tandem with the first ORF encoding the *avrD* gene product [2,4]. Expression of *avrD* within several bacterial species correlates with production of C-glycosyl lipids that can trigger the defense response in *Rpg4* cultivars of soybean, however, no function for this gene (or for the B-plasmid) in the life cycle of the pathogen has yet been identified [5–7].

Originally isolated from *Pst*, four additional *avrD*-related genes have been described in the *phaseolicola*, *lachrymans* and *glycinea* pathovars of *P. syringae* [8], but not in bacteria outside the Pseudomonads. These genes are all plasmid-borne. Here we report that the B-plasmid has been subjected to multiple transposition events. DNA linked to *avrD* shows evidence of class II transpositions and short sequences linked to *avrD* are similar to pathogenicity genes from a variety of unrelated pathogens. Within this region, we also identified a novel IS*3*-related insertion element. Located immediately upstream of *avrD* is the gene cluster that controls B-plasmid stability. In addition, we found DNA sequences that cross-hybridize to *avrD* are also present in the more distantly related soft rot pathogen, *Erwinia carotovora*. Thus, the *avrD* genes may mediate a function that is more general than previously thought. Alternatively, our data are consistent with the possibility that the *avrD* genes may have been transferred horizontally among species.

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2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Escherichia coli DH5α was used as the standard host strain for propagating recombinant plasmids. *P. syringae* pv. *tomato* PT23 [1] and *P. syringae* pv. *glycinea* Race 4 [4] are as described and were provided by Dr. Noel Keen (University of California at Riverside). *E. carotovora* E40 and *Erwinia chrysanthemi* 581 were gifts from Dr. Donald Kobayashi (Rutgers University, New Brunswick, NJ). *Erwinia herbicola* TR1 and *Erwinia amylovora* E266 were gifts from Dr. Don Roth (University of Wyoming, Laramie, WY). *Rhizobium meliloti* IOZF34 was a gift from Dr. Jerry Johnson (University of Wyoming, Laramie, WY).

E. coli strains were routinely grown in Luria-Bertani (LB) medium [9] at 37°C. *Pseudomonas* strains were grown in King's medium B (KMB) [10] or in M9 minimal medium [9] at 28°C. *Erwinia* strains were grown in sucrose peptone agar (20 g sucrose, 5 g peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 15 g agar per liter) at 28°C. *R. meliloti* was grown at 37°C in yeast mannitol broth (YMB) [11].

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Bacterial growth media were obtained from Difco Laboratories (Detroit, MI) or Gibco BRL (Grand Island, NY). Enzymes were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Radioactively labeled nucleotides were obtained from NEN Research Products/DuPont (Wilmington, DE).

2.2. DNA manipulations

Standard recombinant DNA methods were performed as described [9]. DNA of the cosmid clone pPT4E10 (a gift from Dr. Noel Keen, University of California at Riverside) containing *avrD* and adjacent genes was digested with a number of restriction enzymes. The digested DNA was blotted to a nylon filter (Gelman Sciences) and a 5.84 kbp *Bgl*II-*Eco*RI fragment showed hybridization to an *avrD* gene-specific DNA probe. The *Bgl*II-*Eco*RI fragment was recovered from agarose gels via the Geneclean method (Bio101, Inc., Vista, CA) and cloned into pUC118 in both orientations resulting in constructs pTEB11 and pTEB12.

2.3. DNA sequence analysis

Nested deletions were made using the protocol adapted from Henikoff [12]. Ligation products were transformed into *E. coli* DH5α and plasmid DNA was isolated to determine the size of the inserts. Plasmid DNA of selected deletion clones was retransformed into competent cells of *E. coli* MV1193 to prepare single stranded DNA as described previously [13]. DNA sequencing of both strands of the insert was carried out using ^{35}S-radioactively labeled nucleotides

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and Sequenase [14]. Occasionally dITP analogs were used to reduce secondary structure formation. Sequence analysis was carried out using MacVector 4.0.1 and Geneworks sequence analysis programs. In addition, the entire 11 kbp DNA region spanning *avrD* was submitted to the EMBL data base and to GenBank for sequence comparison.

2.4. Genomic Southern blots

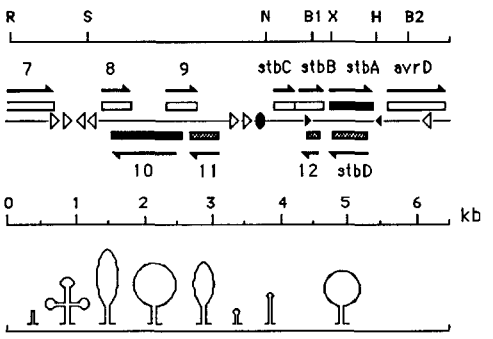
Total DNA of *Rhizobium*, *Pseudomonas*, and *Erwinia* strains was isolated and digested with *Bgl*I or *Hin*dIII, separated on 0.7% agarose gels and blotted onto BioTrace nylon filters (Gelman Sciences) [9]. DNA probes were radioactively labeled with [α-^{32}P]dCTP or [α-^{32}P]dTTP (New England Nuclear) via random priming (BRL random priming kit). Hybridization with ^{32}P-labeled DNA probes were performed in 50% formamide, 5×SSC, 1×Denhardt's solution, 0.02 M sodium phosphate, pH 6.7, and 0.1 mg/ml salmon sperm DNA with gentle shaking at 42°C. Blots were then washed in 2×SSC, 0.1% sodium dodecyl sulfate (SDS) at 42°C for 30 min, followed by 2 washes in 0.1×SSC, 0.1% SDS at 42°C for 30 min before exposure to XAR X-ray film (Kodak). If necessary, filters were washed again in 0.1% SDS, 0.1×SSC for 30 min at elevated temperatures up to a maximum of 68°C.

2.5. GenBank DNA sequence accession number

The DNA sequence upstream of *avrD* has been submitted to GenBank and assigned the accession number L48985.

3. Results

We sequenced 5.84 kbp of DNA that extends upstream from the *Bgl*II site within the *avrD* coding sequence. This region specifies nine ORFs encoding proteins longer than 99 amino acid residues (Fig. 1). A careful comparison of the sequence with DNA sequence data bases predicts functions for five of these ORFs and argues that this region, including *avrD*, has been subjected to multiple transposition events.



I	II	III	IV	V	VI	VII	VIII	Label
573	813	1063	1531	1591	2863	3758	4309	Start
597	1099	3689	5023	3244	2880	3808	5276	Stop
12	38	38	29	30	7	23	26	Stem in bp
1	210	2550	3434	1593	4	4	915	Loop in bp
92	82	76	70	73	100	91	77	% complementarity

Fig. 1. A physical map of DNA sequences linked to *avrD*. Open reading frames are shown as open (upper strand) or hatched (lower strand) boxes. The predicted transposase gene (*tnpA*) and the resolvase gene (*stbA*) are highlighted in black. DNA sequences with significant similarities to terminal inverted repeats of transposable elements are shown as large open triangles. Inverted repeats with sequence similarities to internal repeats of transposons are marked as small black triangles. A sequence highly similar to Tn*3* family promoters is indicated by a black oval. DNA repeat sequences are shown as potential secondary structures. Abbreviations used: R = *Eco*RI, S = *Sal*I, N = *Nco*I, B1 = *Bgl*I, X = *Xho*I, H = *Hin*dIII, B2 = *Bgl*II.

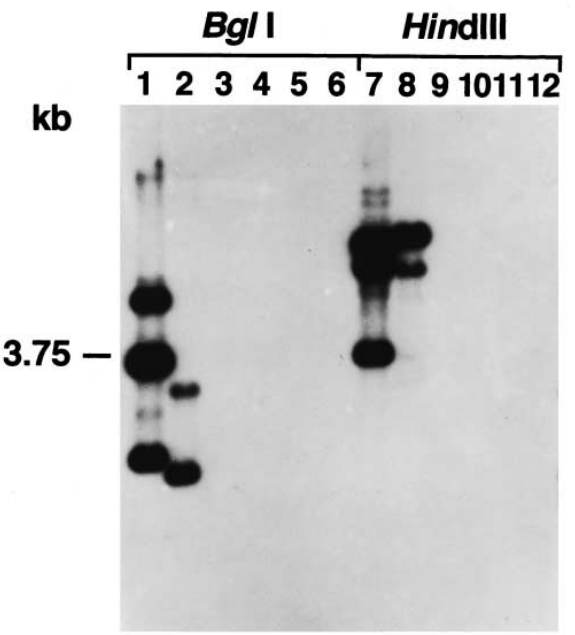


Fig. 2. Multiple copies of IS*1240* are present in *Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *glycinea*. Bacterial genomic DNA (11 μg/lane) was digested with either *Bgl*I (lanes 1–6) or *Hin*dIII (lanes 7–12) and separated on a 0.7% agarose gel. The DNA was transferred to nylon membranes and hybridized with a [^{32}P]DNA probe that spans the entire 1.65 kbp region of the IS*1240* insertion element plus about 500 bp of downstream DNA sequences. The nylon filter was washed in 0.1×SSC, 0.1% SDS for 30 min at 56°C before exposure to X-ray film. The expected size of a hybridizing band (3.75 kbp) of *Pst* PT23 genomic DNA digested with *Bgl*I is indicated on the left. Genomic DNA was isolated from *Pseudomonas syringae* pv. *tomato* PT23 (lanes 1 and 7), *Pseudomonas syringae* pv. *glycinea* Race 4 (lanes 2 and 8), *Erwinia carotovora* E40 (lanes 3 and 9), *Erwinia chrysanthemi* 581 (lanes 4 and 10), *Erwinia herbicola* TR1 (lanes 5 and 11) and *Rhizobium meliloti* IOZF34 (lanes 6 and 12). The intensity of hybridizing bands in lanes 1 and 7 indicates that *P. s.* pv. *tomato* PT23 contains possibly three copies of the insertion element and/or DNA sequences downstream of the insertion element.

3.1. A new class I DNA insertion sequence, IS1240

Insertion sequences (IS) are bounded by imperfect inverted repeat sequences (typically 15–25 bp) that are flanked by a short direct repeat of the host DNA (2–12 bp). This characteristic sequence organization provides prima facie evidence that a given DNA derives from a transposition event [15]. In addition, IS elements commonly encode proteins, such as transposases, that are necessary for mobilization of the element. Class I elements typically carry one gene encoding a transposase (*tnpA*) while the class II elements such as Tn*3* carry two genes required for transposition (*tnpA* and *tnpR*). Based on these criteria, we have identified a new class I insertion element located upstream of *avrD* at position 1587–3248 bp that has been assigned the registration number IS*1240* by the Plasmid Reference Center, Department of Medical Microbiology, Stanford University Medical School, Stanford, CA 94305. The insertion element has a predicted length of 1661 bp including terminal inverted repeats of 32 bp. The left terminal inverted repeat starts at coordinate 1587 bp and shows 69% complementarity to the right terminal inverted repeat which begins at position 3215 bp. The IS is flanked by a 2 bp (GG) target site duplication. An alternative configuration of IS*1240* includes a shorter subset (23 bp) of the left

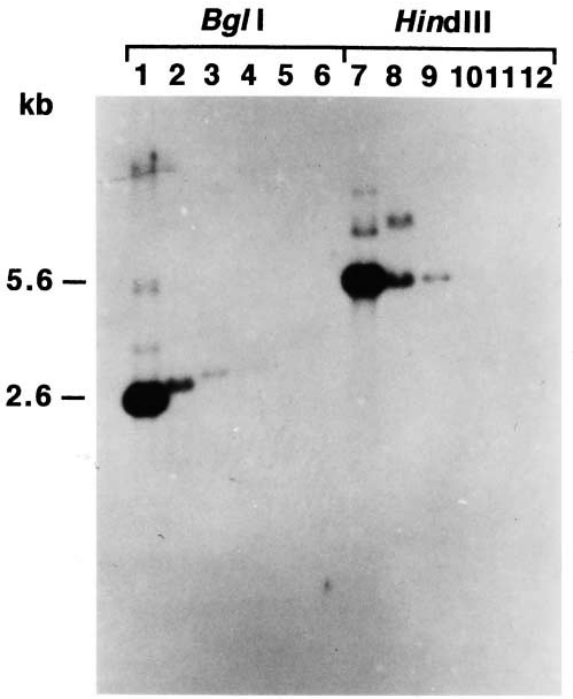


Fig. 3. *AvrD* homologous DNA sequences are present in *Erwinia*. Bacterial genomic DNA (11 μg/lane) was digested with either *Bgl*I (lanes 1–6) or *Hin*dIII (lanes 7–12) and separated on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized to a [^{32}P]DNA probe which spans the *avrD* coding region plus about 150 bp of downstream DNA sequences. The nylon filter was washed in 0.1×SSC, 0.1% SDS for 30 min. at 68°C. Genomic DNA was isolated from *Pseudomonas syringae* pv. *tomato* PT23 (lanes 1 and 7), *Pseudomonas syringae* pv. *glycinea* Race 4 (lanes 2 and 8), *Erwinia carotovora* E40 (lanes 3 and 9), *Erwinia chrysanthemi* 581 (lanes 4 and 10), *Erwinia herbicola* TR1 (lanes 5 and 11) and *Rhizobium meliloti* IOZF34 (lanes 6 and 12). The predicted sizes of hybridizing bands (2.6 and 5.6 kbp) of *P. s.* pv. *tomato* PT23 when digested with *Bgl*I or *Hin*dIII, respectively, are shown in lane 1.

terminal inverted repeat (beginning at position 1593 bp) which would result in increased stability in the stem (83% complementarity compared to 69%). The latter configuration does not include a target site duplication although certain bacterial IS elements do not generate the normal direct repeat upon insertion [16].

Typically more than one copy of a particular insertion sequence is present in the host cell genome [16]. Southern blot hybridizations suggest that at least three copies of IS*1240* exists in *Pst* (Fig. 2). The same experiment also suggests that the related pathogen, *Psg*, contains at least two copies of an IS*1240*-related sequence.

A putative transposase gene (*tnpA*) of 1029 bp is encoded on the lower strand at coordinate 2652–1624 bp beginning with a TTG translational start codon. The presence of TTG as the translational start is consistent with the tendency of transposon-encoded genes to use start codons other than ATG with a high frequency (personal observation based on comparison of GenBank DNA transposase entries). A purine-rich (AGAG) sequence 10 bp upstream of the start codon may function as a ribosome binding site. The translated amino acid sequence of *tnpA* shows significant similarity to a variety of transposase sequences of known insertion elements. For example, the predicted transposase protein shares 60.2% amino acid sequence similarity with the transposase of IS*1141* from *Mycobacterium intracellulare* and it is similar in length [17]. The IS*1240* TnpA is 25.1% identical and 52.6% similar to the shorter (288 amino acids) transposase of *E. coli* IS*3* [18].

3.2. *IS1240-related DNA sequences are present in other plant pathogenic bacteria*

DNA sequences with a high degree of similarity to IS*1240* have been reported in other plant pathogenic bacteria such as *Psg* (Fig. 2). In addition, the pectate lyase gene (*palX*) of *E. carotovora* ssp. *carotovora* is interrupted by an IS*1240*-like DNA fragment encompassing *tnpA* and its upstream sequence [19]. This 1100 bp DNA fragment specifies an amino acid sequence that is 61% identical and 79% similar to the TnpA transposase of IS*1240*.

The pPSP1 plasmid carried by the plant pathogen, *P. s.* pv. *phaseolicola* PK2, contains the ethylene forming enzyme gene, *efe* [20]. A 406 bp DNA sequence located immediately upstream of the *efe* coding sequence shares 87% DNA sequence identity with a portion of the *Pst tnpA* gene, which argues that a fragment of an IS*1240*-like insertion element is located upstream of the *efe* gene.

3.3. *Evidence for multiple class II transposition events in the stb-avrD region of the Pst B-plasmid*

In addition to the class I transposon (IS*1240*), we have identified fragments of class II transposons including similarities to promoter sequences of mercury resistance operons, several terminal inverted repeats and transposon-derived internal inverted repeats (see Fig. 1). For a complete analysis of these terminal inverted repeat sequences and potential stem-loop structures that they might form along with sequence similarities to promoters of mercury resistance operons (see Hanekamp [21]). Taken together, these sequence fragments suggest that the region surrounding the *avrD* gene has survived multiple previous insertions of class II transposons.

3.4. *AvrD-homologous sequence in the soft rot pathogen, Erwinia carotovora*

Transposition activity in the region of *avrD* suggests that the *avrD* gene may have been transferred horizontally among bacterial species. To test this possibility, we isolated genomic DNA from several *Erwinia* and *Pseudomonas* strains as well as from *R. meliloti* and prepared genomic Southern blots. In addition to the expected hybridization signals of *Pst* and *Psg* to the *avrD*-specific DNA probe, we also obtained hybridization signals with genomic DNA from *E. carotovora* E40. Hybridizations, carried out at high stringency (68°C), suggest that *E. carotovora* E40 contains sequences similar to *avrD* (Fig. 3). The calculated melting point of *avrD* under the hybridization conditions that were used is 69°C. The *avrD2* gene in *Psg* is 93% identical to the *avrD* gene of *Pst*. Theoretically *avrD* should not hybridize with *avrD2* under the hybridization conditions that were used unless shorter subsequences of *avrD2* share 100% DNA sequence identity with *avrD*. Since weak hybridization signals of *E. carotovora* with *avrD* can be detected we assume that at least shorter subsequences in *Erwinia* are essentially 100% identical with the *avrD* gene. The hybridizing bands of *Pst, Psg* and *E. carotovora* are similar in size but not identical therefore spill-over of genomic DNA from the *Psg* well to the adjacent well can be excluded. Under conditions of low stringency hybridization (42°C) we also ob-

tained *avrD*-specific hybridization signals to genomic DNA of *E. herbicola*, *E. chrysanthemi* and *R. meliloti* (Hanekamp and Stayton, unpublished observation). Numerous pathogenicity determinants including *hrp*-genes have been described in *Erwinia*. However, to our knowledge, this is the first indication that an *avr*-like gene also may be present in *E. carotovora* E40 and is consistent with horizontal transfer of *avrD* among phytopathogenic bacteria.

3.5. Control of B-plasmid stability in Pst

The DNA sequence located immediately upstream of *avrD* contains a cluster of four ORFs related to plasmid stability loci from several broad host range plasmids. Such loci mediate efficient partitioning of plasmid molecules between daughter cells during cell division. Based on sequence similarities and gene organization, we have designated these ORFs as stability loci, *stbA–D* (Fig. 1).

StbA encodes a DNA resolvase. A search in GenBank as well as in the EMBL data base revealed that the StbA polypeptide shares 70% amino acid sequence identity with the ParA resolvase of the broad host range plasmid RP4 [22]. The ParA resolvase increases plasmid stability by catalyzing monomerization of plasmid multimers thereby increasing the number of plasmid units that can be passed on to daughter cells [23,24]. Like ParA, StbA shares significant amino acid sequence identity with resolvases of the partitioning system of plasmids pAMb1 [25], pIP404 [26,27], and R46 [28] as well as DNA resolvases of transposons of the Tn*3* family, including Tn*3*, Tn*21*, Tnδ, and Tn*501* [29,30]. Significant but reduced sequence similarities also exist between *stbA* and several DNA invertases such as Gin [31], Cin [32], Hin [33] and Bin [34] as well as the plasmid encoded resolvases, ORF 218 (pCf1) and ORF 217 (pCf2) of the eukaryotic diatom *Cylindrotheca fusiformis* [35].

3.6. Other repeated DNA sequences

Certain DNA inversion systems such as Min [36] utilize several inverted repeats in order to carry out multiple DNA sequence inversions. Such gene rearrangements are often used as a means of controlling gene transcription [24,33]. Some characteristic features of DNA inversion systems are present within the region immediately upstream of *avrD*, including two internal repeats flanking the *stb*A resolvase which share similarities with inverted repeats of the Gin, Hin, and Pin DNA inversion systems (reviewed in [31]). These repeats also share similarity with repeats of Tn*21* [37]. Thus, the *stbA* resolvase may, like other resolvases, catalyze DNA inversions at a low frequency. We tested this hypothesis directly, but observed no evidence for StbA-mediated DNA inversion [21]. However, if inversion occurs at low frequency, we could have missed the event.

4. Discussion

4.1. Distribution of avirulence gene D homologs

Based on high stringency Southern blot hybridizations, *E. carotovora* E40 contains DNA sequences that share a significant degree of sequence identity with *avrD*. This is the first report that *avrD*-homologous DNA sequences may exist outside the genus, *Pseudomonas*. The soft rot pathogens, such as *Erwinia*, interact with plants very differently than do the *Pseudomonas* blight and speck pathogens [38]. Thus, the *avrD* genes may have a more general and important function than previously thought, although that role remains unknown. Alternatively, the *avrD*-like sequences in *E. carotovora* E40 may reflect a relatively recent horizontal DNA transfer. Existing data does not permit these models to be distinguished, however, the following observations are suggestive of horizontal transfer of *avrD*.

DNA and protein sequence analysis of the entire 11 kbp *avrD* region not only revealed the presence of a novel insertion element (IS*1240*) but also various DNA sequences (terminal inverted repeats, internal inverted repeats, promoter sequences) that are common to transposable elements. A complex secondary structure and the presence of DNA fragments with similarities to DNA sequences from other animal and plant pathogens suggest that the region surrounding the *avrD* gene has been subject to multiple transposition events and complex rearrangements.

The five 'versions' of the *avrD* gene, that have been sequenced from four pathovars of *P. syringae*, can be grouped into two sequence homology classes [8]. The most divergent pair of *avrD*-related genes are both found in *P. s.* pv. *lachrymans*; each gene carried on a different indigenous plasmid. The *lachrymans* allele 1 and the prototypical *Pst avrD* genes constitute homology class I. The *lachrymans* allele 2 along with the *glycinea* and *phaseolicola avrD* genes form homology group II and are also plasmid-borne. This pattern of distribution is consistent with horizontal gene transfer, perhaps via plasmid conjugation.

Other reports also suggest that transposition and/or horizontal DNA transfer may have been responsible for the wide distribution of avirulence genes. Like the *avrD* gene family, other avirulence genes appear to have highly homologous counterparts in related species. Interestingly, de Feyter and coworkers identified terminal inverted repeats flanking *avrB6* and closely related *avr* genes which suggests that these *avr* genes may have been introduced into *Xanthomonas* via 'recent' transposition events [39]. In addition, the *avrB* and *avrC* genes of *Psg* show a G+C content which led the authors to conclude that these two avirulence genes may have recently been introduced into *Pseudomonas* [40].

4.2. Control of B-plasmid stability in Pst

We have identified the plasmid stability locus of the B-plasmid of *Pst* based on similarities to the plasmid partitioning region of the broad host range plasmid RP4. In contrast to adjacent regions, the *stb* loci appear to be unaffected by transpositions and involve at least four ORFs and a multimer resolution site. The *stb* loci from *Pst* are functional and confer plasmid stability when expressed in *E. coli* [21]. For example, the *stbA* gene encodes a plasmid resolvase that catalyzes a site-specific recombination reaction at the *multimer resolution site* to resolve plasmid dimers to monomers. Despite the close physical linkage (about 200 bp), between the *stbA* and *avrD* coding regions it is not clear if there also exists a functional linkage.

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