A Physical Map of the Syringomycin and Syringopeptin Gene Clusters Localized to an Approximately 145-kb DNA Region of *Pseudomonas syringae* pv. *syringae* Strain B301D

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Genetic and phenotypic mapping of an approximately 145-kb *DraI* fragment of *Pseudomonas syringae* pv. *syringae* strain B301D determined that the syringomycin (*syr*) and syringopeptin (*syp*) gene clusters are localized to this fragment. The *syr* and *syp* gene clusters encompass approximately 55 kb and approximately 80 kb, respectively. Both phytotoxins are synthesized by a thiotemplate mechanism of biosynthesis, requiring large multienzymatic proteins called peptide synthetases. Genes encoding peptide synthetases were identified within the *syr* and *syp* gene clusters, accounting for 90% of the *DraI* fragment. In addition, genes encoding regulatory and secretion proteins were localized to the *DraI* fragment. In particular, the *salA* gene, encoding a regulatory element responsible for syringomycin production and lesion formation in *P. syringae* pv. *syringae* strain B728a, was localized to the *syr* gene cluster. A putative ATP-binding cassette (ABC) transporter homolog was determined to be physically located in the *syp* gene cluster, but phenotypically affects production of both phytotoxins. Preliminary size estimates of the *syr* and *syp* gene clusters indicate that they represent two of the largest nonribosomal peptide synthetase gene clusters. Together, the *syr* and *syp* gene clusters encompass approximately 135 kb of DNA and may represent a genomic island in *P. syringae* pv. *syringae* that contributes to virulence in plant hosts.

Additional keywords: lipopeptide toxin, necrosis-inducing phytotoxin, virulence determinant.

Pseudomonas syringae pv. *syringae* is one of the most abundant bacterial plant pathogens in nature (Bradbury 1986). The bacterium characteristically produces two classes of necrosis-inducing lipopeptide phytotoxins, syringomycins and syringopeptins (Bender et al. 1999; Gross 1991; Iacobellis et al. 1992). Both the syringomycins and syringopeptins are am-

phipathic molecules composed of a hydrophobic 3-hydroxy carboxylic acid tail of varying lengths and a charged cyclic peptide head (Ballio et al. 1995; Fukuchi et al. 1992; Segre et al. 1989; Vaillo et al. 1992). It is the amphipathic nature of these phytotoxins that enables them to insert into membranes and form pores that ultimately lead to plant cell death (Hutchison and Gross 1997).

Recent genetic and biochemical analyses demonstrated that both the syringomycins and syringopeptins are synthesized by a nonribosomal mechanism of peptide biosynthesis (Grgurina and Benincasa 1994; Guenzi et al. 1998; Scholz-Schroeder et al. 2001; Zhang et al. 1995). This mechanism utilizes large multifunctional peptide synthetases composed of one or more modules responsible for the activation and incorporation of specific amino acids into the peptide chain. Genetic analyses of all nonribosomal peptide synthetase systems to date, with the exception of the syringomycin system, have shown them to be arranged in large genomic clusters in which the order and specificity of synthetase modules dictate the peptide sequence (Marahiel et al. 1997; Stein and Vater 1996). Currently, the largest synthetase gene is found in the fungus *Tolytocladium niveum*, which has a large 45.8-kb open reading frame (ORF) that catalyzes cyclosporin A synthesis (Weber et al. 1994). In *P. aeruginosa*, the gene cluster responsible for the nonribosomal peptide synthesis of pyoverdine encompasses a 35-kb region of the 6.3-Mb genome (Lehoux et al. 2000), a size typical for prokaryotic synthetase systems (Marahiel et al. 1997; Moffitt and Neilan 2000). The size of syringomycin (*syr*) and syringopeptin (*syp*) gene clusters is unknown, but they appear to lie adjacent to one another (Scholz-Schroeder et al. 2001), with the *syr* cluster representing a minimum DNA region of 35 kb (Guenzi et al. 1998; Zhang et al. 1995).

Analysis of the *syrB1* and *syrE* genes and the 5' end of the *sypA* gene indicated that they encode peptide synthetases exhibiting a characteristic modular organization (Guenzi et al. 1998; Scholz-Schroeder et al. 2001; Zhang et al. 1995). Each peptide synthetase module encompasses approximately 1,100 amino acids and is encoded by a DNA region of about 3 kb (Marahiel et al. 1997; Stein and Vater 1996). Consequently, nine synthetase modules are associated with syringomycin biosynthesis and are encoded by the *syrB1* and *syrE* genes; ac-

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cordingly, the *syrBI* and *syrE* ORFs together encompass 30.2 kb of DNA (Guenzi et al. 1998; Zhang et al. 1995). The *syrE* ORF alone is 28.4 kb and represents the largest prokaryotic synthetase gene currently found in the GenBank/EMBL database. In addition to biosynthesis genes, genes involved in secretion (i.e., *syrD*) (Quigley et al. 1993) and regulation (i.e., *syrP*) (Zhang et al. 1997) were identified in the *syr* gene cluster. Using the organization of the syringomycin biosynthetic gene cluster as a model, the *syp* gene cluster of *P. syringae* pv. *syringae* strain B301D is predicted to encompass more than 74 kb of DNA. The *syr* and *syp* gene clusters together likely represent over 100 kb of DNA on the chromosome of *P. syringae* pv. *syringae* strain B301D.

It was demonstrated using *syrBI* and *sypA* synthetase mutants that syringomycin and syringopeptin function as virulence determinants in *P. syringae* pv. *syringae* strain B301D (Quigley et al. 1993; Scholz-Schroeder et al. 2001; Xu and Gross 1988a, 1988b; Zhang et al. 1997). Strains carrying mutated synthetase genes were used in virulence studies because they are specifically affected in either syringomycin or syringopeptin biosynthesis. Not only were these synthetase mutants deficient in their ability to produce the respective phytotoxins, but also they were greatly reduced in virulence. In particular, *syrBI-sypA* double mutants failed to produce both syringopeptin and syringomycin and were reduced in virulence by approximately 76% in immature sweet cherry fruit assays. This provided a definitive measure of the contribution of each of the phytotoxins, individually and in combination, to the plant-pathogen interaction (Scholz-Schroeder et al. 2001).

Clamped homogeneous electric field pulsed-field gel electrophoresis (CHEF-PFGE) is an effective tool for the physical mapping of large regions of bacterial genomes (Fonstein and Haselkorn 1995). This technique was used to construct complete physical maps of the *P. aeruginosa* genome (Ratnaningsih et al. 1990; Römmling et al. 1989) and, subsequently, to map the location of virulence-associated genes of *P. aeruginosa* (Shortridge et al. 1991). The *syr* and *syp* gene clusters appear to be located adjacent to one another on the chromosome of *P. syringae* pv. *syringae* strain B301D, based on the recent identification of the *sypA* synthetase gene at the left border of the *syr* gene cluster (Scholz-Schroeder et al. 2001). Preliminary studies suggest that the *syr* and *syp* clusters may be localized to a large *DraI* fragment (approximately 145 kb) resolved by CHEF-PFGE (Caponero et al. 1997; Scholz and Gross 1996). In order to define the functional organization of the gene clusters dedicated to the production of the syringomycin and syringopeptin phytotoxins, we constructed a map of this approximately 145-kb *DraI* fragment by identifying clones from the *DraI* region, generating a physical map of restriction sites, performing insertional mutagenesis at approximately 15-kb intervals, and performing sequence analysis of sites affected by mutagenesis. Phenotypic effects on toxin production and genotypic data on mutagenized regions were used to delineate the *syr* and *syp* gene clusters. Regions carrying genes associated with toxin synthesis, secretion, and regulation were identified, including the map position of the *salA* regulatory gene that was reported by Kitten and colleagues (1998) to be required for syringomycin production and lesion formation by strain B728a of *P. syringae* pv. *syringae*. These results are discussed in regard to the evolution of gene clusters and the relevance to the host-pathogen interaction.

RESULTS

Syringomycin and syringopeptin genes are located on an approximately 145-kb *DraI* fragment.

A single *DraI* fragment, approximately 145 kb, was identified from genomic DNA digests of strain B301D separated by CHEF-PFGE and hybridized with pYM1 (Caponero et al. 1997; Mo and Gross 1991) (Fig. 1), a plasmid carrying a 16-kb *HindIII* fragment composed of both *syr* and *syp* gene sequences (Quigley et al. 1993; Scholz-Schroeder et al. 2001; Zhang et al. 1995, 1997). Using pYM1 in CHEF-PFGE and Southern analysis, two separate DNA fragments, approximately 55 and 90 kb, were identified from genomic DNA of *P. syringae* pv. *syringae* strain BR334 digested with *DraI* (Fig. 1). Strain BR334 is a *syrC* mutant carrying Tn3HoHo1 insert 334 (Quigley et al. 1993; Zhang et al. 1995), possessing an additional *DraI* site located in the transposon. Using *sypA* (pBS008) and *syrE* (pBS006) probes for hybridization, it was determined that *syrE* sequences hybridized to the approximately 55-kb arm and that the *sypA* sequences hybridized to the approximately 90-kb arm of the *DraI* fragment (data not shown). Because mini-Tn5 transposons also contain a *DraI* restriction site, they were used in conjunction with the Tn3HoHo1 insertion in *syrC* of strain BR334 to generate a physical map of the approximately 145-kb *DraI* fragment. In addition to using BR334 carrying the various mini-Tn5 insertions for mapping, these strains served a second purpose. It was observed that *Bacillus megaterium* was inhibited by the production of large quantities of syringomycin (Lavermicocca et al. 1997) and that *sypA* mutants of *P. syringae* pv. *syringae* inhibited the growth of *B. megaterium* (Scholz-Schroeder et al. 2001) due to the production of syringomycin. Therefore, the syringopeptin bioassays were performed on strains of BR334 carrying the various mini-Tn5 insertions. Because strain BR334 does not produce syringomycin, this provided a clear bioassay for syringopeptin.

Most of the syringomycin gene cluster is located on the approximately 55-kb arm of the *DraI* fragment.

Cosmids p149, p634, p29, and p38F2 (Table 1) from a *P. syringae* pv. *syringae* strain B301D genomic library hybridized

Fig. 1. Autoradiograph of p29, p38F2, p116, p149, and p174 DNA hybridizing to genomic DNA isolated from *Pseudomonas syringae* pv. *syringae* strains, digested with *DraI*, and separated using clamped homogeneous electric field pulsed-field gel electrophoresis. Lane A, B301DR (parental); lane B, BR334 (*syrC* mutant); lane C, BR334.5; lane D, BR334.1; lane E, BR334.3; lane F, BR334.2; lane G, BR334.5; and lane H, BR334.6.

to the approximately 55-kb arm of the *DraI* fragment from *P. syringae* pv. *syringae* strain BR334 (data not shown). Restriction maps were constructed for each of the cosmids using *DraI*, *HindIII*, *EcoRI*, and *KpnI* (Fig. 2). Comparison of the restriction maps combined with Southern hybridization demonstrated that p29 and p634, and pYM1 and p149, carried overlapping DNA (Fig. 2). In addition, p2395, carrying a 13-kb *EcoRI* fragment with a Tn5 insert isolated from syringomycin mutant strain W4S2395 (Xu and Gross 1988a), was shown to hybridize to the approximately 55-kb arm of the *DraI* frag-

ment and overlap cosmids p29 and p38F2. A subgenomic clone, pBS025, was identified to overlap pYM1 and p38F2. A *DraI* restriction site was identified in p634 corresponding to the end of the approximately 55-kb arm of the *DraI* fragment. Cosmids p149, p634, p29, and p38F2 together with plasmids pYM1, p2395, and pBS025 encompass the entire approximately 55-kb arm of the approximately 145-kb *DraI* fragment.

Several regions along the approximately 55-kb arm were subsequently subcloned, mutagenized, and recombined back into the genome of strains B301D and BR334 for genotypic

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
B301D	Wild type from pear	Cody et. al. 1987
B301DR	Spontaneous Rif ^r derivative of B301D	Xu and Gross 1988b
BR334	<i>syrC::Tn3HoHo1</i> derivative of B301DR; Pip ^r Rif ^r	Quigley et al. 1993
B301D.1	mini-Tn5 derivative of B301D from pBS042Km; Km ^r	This study
B301D.2	mini-Tn5 derivative of B301D from pBS044Km; Km ^r	This study
B301D.3	mini-Tn5 derivative of B301D from pSL008Km; Km ^r	This study
B301D.4	mini-Tn5 derivative of B301D from pJS013Km; Km ^r	This study
B301D.5	mini-Tn5 derivative of B301D from pJS049Km; Km ^r	This study
B301D.6	mini-Tn5 derivative of B301D from pJS055Km; Km ^r	This study
BR334.1	mini-Tn5 derivative of BR334 from pBS042Km; Km ^r Pip ^r Rif ^r	This study
BR334.2	mini-Tn5 derivative of BR334 from pBS044Km; Km ^r Pip ^r Rif ^r	This study
BR334.3	mini-Tn5 derivative of BR334 from pSL008Km; Km ^r Pip ^r Rif ^r	This study
BR334.4	mini-Tn5 derivative of BR334 from pJS013Km; Km ^r Pip ^r Rif ^r	This study
BR334.5	mini-Tn5 derivative of BR334 from pJS049Km; Km ^r Pip ^r Rif ^r	This study
BR334.6	mini-Tn5 derivative of BR334 from pJS055Km; Km ^r Pip ^r Rif ^r	This study
<i>Escherichia coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ <i>lacX74</i> (ϕ 80 Δ <i>lacZ</i> Δ M15) Δ (<i>mrr-hsdRMS-mcrB</i>) <i>deoR recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ^- <i>rpsL nupG</i>	Hanahan 1985
XL1-Blue MR	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1</i> [F ⁺ <i>proAB lacI</i> ^q Δ M15 Tn10 (Tet ^r)]	Stratagene, La Jolla, CA, U.S.A.
Plasmids		
pBluescript II SK	High-copy-number cloning vector; Ap ^r	Stratagene
pUC18	High-copy-number cloning vector; Ap ^r	Yanisch-Perron et al. 1985
pUTKm	Delivery plasmid for mini-Tn5 Km; Km ^r	Herrero et al. 1990
pBR325	Low-copy-number plasmid for marker exchange; Tc ^r	Prentki et al. 1981
pLAFR3	IncP <i>cos⁺</i> <i>rlx⁺</i> ; Tc ^r	Staskawicz et al. 1987
pYM1	pGS72 carrying a 16-kb <i>HindIII</i> fragment from strain B301D; Tc ^r	Mo and Gross 1991
pBS006	pUC18 carrying a 3-kb <i>BamHI</i> fragment of p38F2; Ap ^r	This study
pBS008	pUC18 carrying a 5.1-kb <i>EcoRI-KpnI</i> fragment of pYM1; Ap ^r	Scholz-Schroeder et al. 2001
p2395	pBR325 carrying 13-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA; Tc ^r	D. C. Gross, <i>personal communication</i>
pJS013	pBSK carrying a 7.8-kb <i>EcoRI</i> fragment of p753; Ap ^r	This study
pJS049	pBSK carrying an 8.1-kb <i>BamHI</i> fragment of p174; Ap ^r	This study
pJS055	pBSK carrying a 2.1-kb <i>HindIII</i> fragment of p116; Ap ^r	This study
pJS013Km	pJS013 with mini-Tn5 inserted into <i>BstZ17</i> site moved to pBR325; Km ^r Tc ^r	This study
pJS049Km	pJS049 with mini-Tn5 inserted into <i>NsiI</i> site moved to pBR325; Km ^r Tc ^r	This study
pJS055Km	pJS055 with mini-Tn5 inserted into <i>BstEII</i> site moved to pBR325; Km ^r Tc ^r	This study
pBS042	pUC18 carrying a 3.0-kb <i>BamHI</i> fragment of p38F2; Ap ^r	This study
pBS044	pUC18 carrying a 3.2-kb <i>EcoRI/HindIII</i> fragment of p2395; Ap ^r	This study
pBS042Km	pBS042 with mini-Tn5 inserted into <i>NsiI</i> site moved to pBR325; Km ^r Tc ^r	This study
pBS044Km	pBS014 with mini-Tn5 inserted into <i>BpoI</i> site moved to pBR325; Km ^r Tc ^r	This study
pSL008	pBR325 carrying a 3.0-kb <i>EcoRI</i> fragment of p29; Tc ^r	This study
pSL008Km	pSL008 with mini-Tn5 inserted into <i>KpnI</i> site moved to pBR325; Km ^r Tc ^r	This study
pJS091	pBSK carrying a 4.7-kb <i>HindIII</i> fragment from strain B301D; Ap ^r	This study
pBS025	pUC18 carrying a 12-kb <i>BamHI/EcoRI</i> fragment from strain B301D; Ap ^r	This study
pJS115	pBSK carrying an 8.4-kb <i>KpnI</i> fragment from strain B301D; Ap ^r	This study
Cosmids		
p29	pLAFR3 carrying a 16-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p38F2	pLAFR3 carrying a 19-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p27B5	pLAFR3 carrying a 20-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p116	pLAFR3 carrying a 20-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p149	pLAFR3 carrying a 30-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p174	pLAFR3 carrying a 23-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p634	pLAFR3 carrying a 24-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study
p753	pLAFR3 carrying an 8-kb <i>P. syringae</i> pv. <i>syringae</i> strain B301D genomic DNA fragment; Tc ^r	This study

^a Ap^r, Km^r, Pip^r, Tc^r, and Rif^r = resistance to ampicillin, kanamycin, piperacillin, tetracycline, and rifampicin, respectively.

Sequence analysis of 500 bases upstream and downstream of the mini-Tn5 insertions carried on pBS042Km and pBS044Km demonstrated that mini-Tn5 insertions 1 and 2 were located within the *syxE* gene (GenBank/EMBL accession no. AF047828) at nucleotides 4,112 and 23,737, respectively (Table 2, Fig. 2). Consequently, mini-Tn5 insertions 1 and 2 are located in *syxE* regions encoding modules 2 (serine) and 8 (3-hydroxy aspartic acid) of the SyxE synthetase (Guenzi et al. 1998). Sequence analysis of the Tn5 insert carried on plasmid p2395 determined that it is located at nucleotide 27,295 of *syxE*, right at the beginning of the terminal thioesterase do-

Fig. 2. Diagrammatic representation of the approximately 145-kb *DraI* fragment of *Pseudomonas syringae* pv. *syringae* strain B301D. The syringopeptin (red arrow) and syringomycin (blue arrow) gene clusters are presented with their relative location on the approximately 145-kb *DraI* fragment. The position and orientation of the *sydD*, *sydP*, *sydB1*, *sydB2*, *sydC*, *sydE*, and *sydA* genes as organized on the *P. syringae* pv. *syringae* B301D chromosome are inset above the map of the approximately 145-kb *DraI* fragment. The placement of the cosmids and subgenomic clones along the *DraI* fragment are indicated below the map as solid black and diagonally hatched lines, respectively. The position of the Tn3HoHo1 insert 334 located within *sydC* is designated with a horizontal dotted line. Vertical arrows numbered 1 through 6 indicate the positions of the mini-Tn5 insertions used to map the *DraI* fragment. The phenotypic results of insertions along the 145-kb *DraI* fragment are designated with a blue flag pointed right for loss of syringomycin production; a red flag pointed left for loss of syringopeptin production; and a double-sided black flag for loss of syringomycin and syringopeptin production. The restriction enzyme sites for *EcoRI* (E), *HindIII* (H), *KpnI* (K), and *DraI* are identified.

main of the SyrE synthetase (Guenzi et al. 1998). It was also determined that the mini-Tn5 insertion 3, carried on pSL008Km, is located at nucleotide 686 within the *salA* gene (GenBank/EMBL accession no. AF022808) (Kitten et al. 1998) (Table 2). The *salA* gene was originally described from *P. syringae* pv. *syringae* strain B728a, and we estimate it to be located approximately 8 kb downstream of the *syrE* synthetase gene in strain B301D. Six nucleotide differences were observed after comparison of the last 669 nucleotides (Kitten et al. 1998) obtained from the *salA* gene from strain B301D with the corresponding 669 nucleotides of the *salA* gene from strain B728a. Furthermore, a single amino acid difference was observed by comparison of the putative SalA amino acid sequence of strain B301D to the analogous sequence of SalA from strain B728a (Kitten et al. 1998).

The syringopeptin gene cluster is located on the approximately 90-kb arm of the *DraI* fragment.

Four cosmids, p149, p753, p174, and p116, isolated from a *P. syringae* pv. *syringae* strain B301D genomic library hybridized to the original approximately 145-kb *DraI* fragment and to the approximately 90-kb *DraI* fragment in strain BR334 (data not shown). Cosmids were mapped by restriction analysis using *DraI*, *HindIII*, *EcoRI*, and *KpnI* (Fig. 2). Southern hybridization identified overlaps that were present between p149 and pYM1, as well as between p753 and p174. Chromosomal walking using p116 sequences as a hybridization probe identified strain B301D genomic subclone pJS091 (Fig. 2, Table 1). Repeating this step, using pJS091 as a hybridization probe, genomic subclone pJS115 of strain B301D was identified (Fig. 2, Table 1). Using pJS115 as a hybridization probe, cosmid p27B5 was identified from the genomic library. These cosmids and plasmids were mapped by restriction analysis using *DraI*, *HindIII*, *EcoRI*, and *KpnI* (Fig. 2). A *DraI* restriction site was identified in cosmid p27B5, demonstrating that it carried sequences located at the end of the approximately 90-kb arm of the approximately 145-kb *DraI* fragment.

In order to map the approximately 90-kb arm of the *DraI* fragment, regions were chosen from the various cosmids and plasmids spanning the region for subcloning and mutagenesis. Restriction fragments were chosen for subcloning from p753, p174, and p116 and mutagenized by the site-specific introduction of mini-Tn5 insertions to construct plasmids pJS013Km, pJS049Km, and pJS055Km, respectively (Table 1). Strains B301D.4, B301D.5, and B301D.6, as well as BR334.4, BR334.5, and BR334.6, were generated by homologous recombination of the mutagenized fragments carried on plasmids pJS013Km, pJS049Km, and pJS055Km, respectively, into strains B301D and BR334, generating mini-Tn5 insertions 4, 5, and 6 (Fig. 2). CHEF-PFGE and Southern analysis indicated that the mini-Tn5 insertions 4, 5, and 6 mapped approximately 40, 50, and 85 kb, respectively, from Tn3HoHo1 insert 334 (Fig. 2).

To further characterize mini-Tn5 insertions 4, 5, and 6, the respective strains carrying the insertions were analyzed in phytotoxin bioassays and the insertions were sequenced. Strains carrying mini-Tn5 insertions 4 and 5 produced wild-type amounts of syringomycin, based on inhibition of *G. candidum* growth; however, no syringomycin production was detected for strains carrying mini-Tn5 insertion 6. In contrast, strains carrying mini-Tn5 insertions 4, 5, and 6 did not pro-

duce zones of inhibition against *B. megaterium*, which indicates a lack of syringopeptin production (data not shown). Wild-type levels of inhibition were observed for B301D.6 complemented *in trans* with the parental cosmid p116. In addition, it is hypothesized that the *syp* gene(s) could be as large as *syrE* (28.4 kb) and it is not expected that an entire *syp* gene would be located on a single cosmid from a genomic library. Therefore, as expected, wild-type levels of inhibition were not observed for B301D.4 and B301D.5 complemented *in trans* with the parental cosmids p753 and p174, respectively. Sequence analysis of DNA flanking the mini-Tn5 insertions 4 and 5 identified sequences encoding peptide synthetases. BLAST analysis demonstrated that the putative protein products are homologous to SyrE (GenBank/EMBL accession no. AF047828) (Table 2). BLAST analysis of the DNA flanking mini-Tn5 insertion 6 indicated that it is located within a gene encoding a protein with homology to YbjZ (GenBank/EMBL accession no. AE000189), an ABC transporter (Blattner et al. 1997) (Table 2). Mini-Tn5 insertion 6 apparently disrupts a gene whose putative protein product may be involved in the secretion of both phytotoxins.

DISCUSSION

Two nonribosomal peptide synthetase systems, the *syr* and *syp* biosynthetic gene clusters, were localized to an approximately 145-kb *DraI* fragment. Phenotypic and genotypic analyses of this *DraI* fragment identified important aspects unique to phytotoxin production by *P. syringae* pv. *syringae*: (i) the *syr* and *syp* biosynthetic clusters are distinct and located adjacent to each other on the B301D chromosome; (ii) a minimum of 66% of the *DraI* fragment encodes peptide synthetases responsible for syringomycin and syringopeptin biosynthesis; (iii) genes involved in the regulation (e.g., *salA*) and secretion (e.g., ABC transporter homolog) of syringomycin and syringopeptin are located at the right and left border regions of the clusters, respectively; and (iv) the *syr* and *syp* gene clusters account for 93% of the *DraI* fragment and, therefore, comprise approximately 2% of the *P. syringae* pv. *syringae* genome (based on a genome size range of 3.7 to 7.1 Mb for *Pseudomonas* species [Spiers et al. 2000; Stover et al. 2000]). The *syr* and *syp* gene clusters combined are the largest known DNA region dedicated to nonribosomal peptide synthetase systems in a microorganism, and they appear to constitute a genomic island (Hacker and Kaper 2000) of approximately 135 kb in *P. syringae* pv. *syringae*.

The *syp* gene cluster and nearly the entire *syr* gene cluster are located on the approximately 145-kb *DraI* fragment. The *syp* gene cluster encompasses approximately 80 kb of DNA, and approximately 70 kb of DNA from this region is dedicated to encoding peptide synthetases. In addition, mutations located in regions coding for peptide synthetases disrupted either syringomycin or syringopeptin production. This indicates that syringomycin and syringopeptin are synthesized by separate nonribosomal peptide synthetase systems. There are several nonribosomal peptide synthetase systems in which large gene clusters are responsible for the synthesis of a peptide. The largest peptide synthetase, CYSYN, is encoded by the 45.8-kb *simA* gene (Weber et al. 1994). CYSYN is responsible for the activation and incorporation of 11 amino acids during the nonribosomal synthesis of cyclosporin A in the fungus *T.*

niveum. In addition, a 35-kb gene cluster containing several peptide synthetase genes was also identified in *P. aeruginosa* (Lehoux et al. 2000). This gene cluster is responsible for the nonribosomal peptide synthesis of pyoverdine, resulting in the incorporation of eight amino acids into the peptide structure. It was previously reported that the *syr* gene cluster was located on the large arm of the approximately 145-kb *DraI* fragment (Caponero et al. 1997). Considering the facts that *sypA* gene sequences hybridize to the large arm of the *DraI* fragment and that insertions 5 and 6 map to the large arm of the *DraI* fragment and specifically disrupt syringopeptin production, it is evident that the *syr* gene cluster is located on the small arm of the approximately 145-kb *DraI* fragment. However, it was determined that the *syr* gene cluster phenotypically extends to, and may extend past, the *DraI* site. Consequently, the *syr* gene cluster currently encompasses approximately 55 kb of DNA, 30.2 kb of which is dedicated to encoding syringomycin peptide synthetases (Guenzi et al. 1998). The *syr* and *syp* gene clusters were phenotypically delineated to encompass approximately 135 kb of DNA, and it is evident that a great proportion of the cluster (approximately 78%) is dedicated to phytotoxin biosynthesis.

Peptide synthetases contain conserved, repetitive modules composed of condensation, amino acid activation, and thiolation domains (Marahiel et al. 1997). Approximately 3 kb of DNA is required to encode a module, and a separate module is required for the addition of each amino acid to the peptide. Contiguous cosmids and plasmids were not identified that encompassed the entire large arm of the *DraI* fragment (Fig. 2). However, the DNA encoding the first module of syringopeptin biosynthesis, SypA-M1, previously sequenced and analyzed (Scholz-Schroeder et al. 2001), were mapped to the large arm of the *DraI* fragment. The conserved structure of peptide synthetases (Marahiel et al. 1997), in conjunction with the CHEF-PFGE data, enabled estimations to be made with regard to the map of the *syp* gene cluster (Fig. 2). The current physical map of the *syp* gene cluster accounts for the physical distance required for syringopeptin biosynthesis and supports the biosynthesis of syringopeptin by nonribosomal peptide synthetases (Grgurina and Benincasa 1994; Scholz-Schroeder et al. 2001).

The right border of the *syr* gene cluster contains genes that strongly affect syringomycin production. The most prominent observation of this region was the mapping of the *salA* gene to the *syr* gene cluster near the end of the approximately 145-kb *DraI* fragment. The *salA* gene was originally described by Kitten and colleagues (1998) from *P. syringae* pv. *syringae* strain B728a to have an important role in the plant-pathogen interaction. The *salA* gene is a component of the *gacA/gacS* regulon that regulates both syringomycin production and the ability to form lesions on bean leaves (Kitten et al. 1998). Although this gene was functionally linked to syringomycin production, its location relative to the *syr* gene cluster was undefined. Here, we report that it is located approximately 10 kb from the end of the *DraI* fragment and approximately 8 kb from the end of *syrE*. In addition, we found the *salA* gene sequence attained from *P. syringae* pv. *syringae* strain B301D to be essentially identical to that of strain B728a. The *salA* gene is the second regulatory gene identified within the *syr* gene cluster to have a role in the production of syringomycin. The *syrP* gene, located between the *syrB1* and *syrD* genes, was previously shown to have an effect on syringomycin produc-

tion in response to phosphate levels (Zhang et al. 1997). The SyrP protein exhibits homology to proteins such as CheA and KinA that are regulatory elements in phosphorelay pathways. The identification of a second regulatory gene suggests that there are multiple levels of regulation for syringomycin. In addition, it is evident that the regulation of syringomycin and syringopeptin is separate. The identification of the *salA* gene within the *syr* gene cluster suggests that genes other than peptide synthetases have an important role in the production of syringomycin by *P. syringae* pv. *syringae* strain B301D.

The left border of the *syp* gene cluster contains a gene that may affect the secretion of both syringomycin and syringopeptin. A putative ABC transporter homolog is located within the *syp* cluster about 10 kb from the *DraI* restriction site. Mutagenesis of this region results in the loss of syringopeptin and syringomycin production. This phenotype is similar to a previously identified ABC transporter homolog, SyrD (Quigley et al. 1993), which is responsible for the secretion of both syringomycin and syringopeptin (Grgurina et al. 1996). It is apparent that the *syp* and *syr* clusters are phenotypically linked with regard to the secretion of the two toxins, even though *syrD* and the newly identified putative ABC transporter homolog are physically separated by more than 75 kb of DNA. The *P. aeruginosa* genome contains many (>20) currently undescribed efflux systems including ABC transporters (Stover et al. 2000), and it was suggested that efflux systems could have wide-ranging specificity and be responsible for the secretion of multiple substrates (Van Bambeke et al. 2000). It is possible that there are two or more separate secretion systems responsible for the secretion of both syringomycin and syringopeptin.

The concept of genome evolution and how gene clusters contribute to the adaptive fitness of an organism is the focus of intense discussion and debate (Hacker and Kaper 2000; Lawrence and Roth 1996). Hacker and Kaper (2000) described genomic islands as large contiguous DNA regions (10 to 200 kb) whose gene products contribute to the adaptation and fitness of both pathogenic and nonpathogenic bacterial strains. These genomic islands may or may not contain characteristic features of pathogenicity islands, and pathogenicity islands themselves are a subset of genomic islands. The approximately 135 kb of DNA encompassed by the *syr* and *syp* gene clusters accounts for about 2% of the *P. syringae* pv. *syringae* strain B301D genome (based on a genome size of 6.26 Mb for the *P. aeruginosa* PA01 genome [Stover et al. 2000]). In addition, the production of syringomycin and syringopeptin is a stable phenotype for *P. syringae* pv. *syringae* strain B301D (Quigley and Gross 1994). However, using pYM1 carrying *syr* and *syp* gene sequences as a hybridization probe (Fig. 2), it was demonstrated that this region is unstable in *P. syringae* pv. *syringae* strain B728a (Rich and Willis 1997). In addition, the *syr* and *syp* gene clusters are not present in other pathovars of *P. syringae* (Quigley and Gross 1994). The presence of the *syr-syp* gene clusters may be the result of a macroevolutionary process leading to *P. syringae* pv. *syringae* and other closely related pathovars producing lipodepsipeptide phytotoxins (Hacker and Kaper 2000). It is evident from this analysis of *P. syringae* pv. *syringae* mutants (Quigley et al. 1993; Xu and Gross 1988b; Zhang et al. 1995) that disruption of one or both of the toxins can occur by mutagenesis in numerous places along the approximately 145-kb *DraI* fragment, suggesting that strict

conservation of the DNA sequence in the *syr-syp* cluster is necessary for the production of both phytotoxins in *P. syringae* pv. *syringae*. It was previously demonstrated that both syringomycin and syringopeptin together contribute extensively to the virulence of the *P. syringae* pv. *syringae* strain B301D, with virulence of *syrB1-sypA* double mutants reduced by approximately 76% in immature sweet cherry fruit assays (Scholz-Schroeder et al. 2001). While it is evident they function as virulence determinants, it is unclear what other roles, if any, these phytotoxins may play in the fitness of *P. syringae* pv. *syringae*. Consequently, it appears that the *syr-syp* gene clusters may represent a genomic island (Hacker and Kaper 2000).

This is not the first report of a genomic island within *P. syringae* pv. *syringae*. The *Hrp* pathogenicity island of *P. syringae* pv. *syringae* is composed of the *hrp/hrc* gene cluster flanked by the exchangeable effector locus and the conserved effector locus (Alfano et al. 2000). Together, the tripartite pathogenicity island, a subset of a genomic island (Hacker and Kaper 2000), contributes to the pathogenicity and parasitic fitness of *P. syringae* pv. *syringae*. Recently, Lawrence and Roth (1996) described a model for the transfer of gene clusters that contribute to the fitness of the organism and designated them selfish operons. A conceptual picture of the *syr* and *syp* gene clusters is developing, and it may become evident that they are genomic islands and may function as selfish operons.

Genotypic and phenotypic analyses of the 145-kb *DraI* fragment carrying the *syr* and *syp* gene clusters provide a valuable foundation for the analysis of syringomycin and syringopeptin production in *P. syringae* pv. *syringae* strain B301D. The delineation of the syringopeptin biosynthetic region helps facilitate sequencing of this region and the identification of gene(s) involved in syringopeptin biosynthesis. In addition to genes coding for peptide synthetases, it would be expected that there may be genes coding for epimerization of amino acids within the peptide structure, as well as acyl-transferase activity responsible for the attachment of the fatty acid tail to the peptide (Marahiel et al. 1997). The demarcation of the *syr* and *syp* gene clusters and their constituents as a genomic island continues to further our understanding of the role of the *syr* and *syp* gene clusters not only to the biology of the organism, but also to the evolutionary development of *P. syringae* pv. *syringae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

Bacterial strains and plasmids are listed in Table 1. *P. syringae* pv. *syringae* strains were routinely cultured at 25°C in nutrient broth yeast extract (NBY) liquid or agar media (Vidaver 1967). *Escherichia coli* strains DH10B (Gibco-BRL Life Technologies, Inc., Grand Island, NY, U.S.A.) and XL1-Blue MR (Stratagene, La Jolla, CA, U.S.A.) were cultured at 37°C in Luria Bertani (LB) broth or agar (Sambrook et al. 1989). Syringomycin bioassays were performed on potato dextrose agar (PDA) supplemented with 0.4% casamino acids and 1.5% glucose (20 ml per plate) (Gross and DeVay 1977). The syringopeptin bioassays were performed on peptone-glucose-NaCl agar (PGNA) containing 5 g of peptone, 10 g of glucose, 5 g of NaCl, and 8 g of agar (20 ml per plate) (Bultreys and Gheysen 1999). Antibiotics (Sigma Chemical Co., St. Louis) were added as required to media using the following final con-

centrations: ampicillin, 100 µg/ml; tetracycline, 25 µg/ml; piperacillin, 25 µg/ml; and kanamycin, 100 µg/ml.

Bioassay for syringomycin and syringopeptin production by *P. syringae* pv. *syringae* strains.

Each of the *P. syringae* pv. *syringae* strains used in this study was screened for the ability to produce syringomycin and syringopeptin using the PDA and PGNA plate bioassays described by Scholz-Schroeder and colleagues (2001). Briefly, *P. syringae* pv. *syringae* strains were grown overnight in 5 ml of NBY liquid medium. Bacterial cells were harvested by centrifugation and washed once with sterile distilled water (SDW). The cells were resuspended in SDW to an optical density of 0.3 (approximately 2×10^8 CFU/ml), and 5-µl aliquots of bacterial suspension were spotted onto PDA and PGNA plates. The plates were incubated for 3 days at 25°C, and PDA plates were oversprayed lightly with an arthrospore suspension of the bioassay fungus, *G. candidum* F-260, whereas PGNA plates were oversprayed with a cell suspension of *B. megaterium* strain Km (Grgurina et al. 1996; Lavermicocca et al. 1997). The *P. syringae* pv. *syringae* mutant strains were compared with the parental strain for the formation of zones of fungal and bacterial inhibition. To avoid confusion in distinguishing between syringomycin and syringopeptin zones of inhibition, mutations were introduced into strains B301D and BR334 (Zhang et al. 1995). Strain BR344 is a syringomycin *syrC* mutant of B301DR that produces large amounts of syringopeptin (Grgurina et al. 1996), which allows for a distinct bioassay for syringopeptin.

DNA manipulations and sequence analysis.

Plasmid DNA were isolated using a cetyltrimethylammonium bromide boiling plasmid preparation (Del Sal et al. 1989). Routine procedures (Ausubel et al. 1990; Sambrook et al. 1989) were used for cosmid and genomic DNA isolation, restriction endonuclease digestions, and subcloning.

Sequencing primers were synthesized by Operon Technologies, Inc. (Alameda, CA, U.S.A.). Sequence reactions were completed for both strands using fluorescence-based dideoxy terminators and Ampli-Taq polymerase (Perkin-Elmer Applied Biosystems, Inc., Norwalk, CT, U.S.A.). Sequence reactions were run on an ABIPRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Inc.). Sequence data were analyzed using the Wisconsin Sequence Analysis programs of the Genetic Computer Group (GCG) (release beta 10.0; Genetic Computer Group, Inc., Madison, WI, U.S.A.) (Devereux et al. 1984). The program NETBLAST (Altschul et al. 1990) was used to identify nucleic acid and protein homologies. The program BESTFIT was used for the comparison of two protein sequences and Z-score calculations. The BESTFIT program was run using the randomization option of 100 to produce the data necessary for Z-score calculations. The Z score was calculated using the actual quality score minus the mean quality value of the randomized scores divided by the standard deviation of randomized score distribution. Z scores greater than six were considered significant.

Cosmid library construction.

Chromosomal DNA from *P. syringae* pv. *syringae* strain B301D were prepared using standard techniques (Ausubel et al. 1990; Sambrook et al. 1989). The chromosomal DNA were

partially cleaved using *Sau3A* (Gibco-BRL Life Technologies, Inc.) and size-fractionated by sucrose gradient centrifugation as described by Sambrook and colleagues (1989). DNA ranging from 16 to 30 kb were ligated into cosmid vector pLAFR3 after *Bam*HI digestion and alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, U.S.A.) treatment. Recombinant cosmids were packaged using Gigapack II packing extracts (Stratagene) and then used to infect *E. coli* strain XL1-Blue MR according to the manufacturer's instructions; α -complementation was used to select colonies harboring an insert. The library was amplified using standard procedures (Sambrook et al. 1989). Approximately 1,000 colonies were selected from the primary library and 3,000 colonies were selected from the amplified library representing approximately 10 genome equivalents. All colonies were individually picked into separate microtiter wells containing 100 μ l of LB broth plus tetracycline. The microtiter plates were incubated overnight at 37°C as still cultures. The next day, 10- μ l aliquots were used to reinoculate a microtiter plate containing 100 μ l of fresh LB plus tetracycline and incubated overnight at 37°C to ensure uniform growth. A 10- μ l aliquot of each well was spotted in duplicate onto GeneScreen membrane filters (NEN Life Science Products, Boston, MA, U.S.A.), placed on LB agar plus tetracycline, and incubated overnight at 37°C. The resultant colonies were lysed with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralized (0.5 M Tris-HCl, pH 7.2; 1.5 M NaCl; 100 mM EDTA). The DNA were fixed to the membrane by baking at 80°C for 2 h under a vacuum. Glycerol (50 μ l of sterile 80% stock) was added to the remaining 130 μ l of culture in each well of the microtiter plates, mixed, and stored at -80°C.

CHEF-PFGE.

The procedures used for isolation, digestion, and electrophoresis of *P. syringae* pv. *syringae* genomic DNA were modified from work by Rainey and colleagues (1993). *P. syringae* pv. *syringae* strains were grown overnight in 5 ml of NBY liquid medium. Cells were harvested by centrifugation and washed twice in sodium chloride-ethylenedinitrilo tetraacetic acid-Tris buffer (NET 100) buffer (100 mM NaCl; 100 mM EDTA; 10 mM Tris-HCl, pH 8.0). To form agarose plugs, the washed cells were resuspended in 1 ml of NET 100, mixed with an equal volume of 2% (wt/vol) chromosomal grade agarose, prepared in NET 100, and aliquoted into well molds. The *P. syringae* pv. *syringae* cells suspended in agarose were then placed in a lysis solution (6 mM Tris-HCl, pH 7.6; 1 M NaCl; 100 mM EDTA, pH 8.0; 0.5% sarkosyl; 1 mg of lysozyme per ml) at 37°C for 24 h, followed by a second lysis solution (0.5 M EDTA, pH 9.0; 1% sarkosyl; 1.5 mg of proteinase K per ml) incubated at 50°C for 48 h. The agarose blocks were finally treated with 50 mM phenylmethylsulfonyl fluoride for 2 h to inhibit proteinase K activity and stored in 0.5 M EDTA at 4°C. DNA plugs were placed in 1 $\times$ Tris-EDTA (TE) (pH 7.6) for 12 h (overnight) at 4°C, transferred to fresh 1 $\times$ TE (pH 7.6), and incubated for 1 h at room temperature. DNA plugs were then equilibrated twice with 1 $\times$ restriction buffer at room temperature for 1 h. The 1 $\times$ restriction buffer was replaced with 1 ml of fresh 1 $\times$ restriction buffer and the DNA, suspended in agarose plugs, were digested (37°C for 24 h) using 20 U of *Dra*I restriction endonuclease (Gibco-BRL Life Technologies, Inc.).

Agarose blocks containing digested *P. syringae* pv. *syringae* DNA were loaded onto a 1.0% agarose, 0.5 $\times$ Tris-borate-EDTA (TBE)-buffered gel and run on a CHEF DRII pulsed-field electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) containing 0.5 $\times$ TBE buffer cooled to 5°C. A ramped pulse time of 5 to 10 s over 20 h at 175 V was used to resolve the DNA fragments. Gels were stained in ethidium bromide (10 μ g/ml) for 30 min at room temperature and destained in SDW for 1 h before visualization. Sizes were estimated by comparison with comigrating fragments of lambda-ladder PFG and low-range PFG marker (New England Biolabs, Beverly, MA, U.S.A.). The resultant gel was transferred to a Hybond membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.) for Southern analysis using standard procedures (Sambrook et al. 1989).

Identification and characterization of cosmids that hybridize to the approximately 145-kb *Dra*I fragment.

The B301D genomic library was screened using the approximately 145-kb *Dra*I fragment excised from a CHEF-PFGE gel as a hybridization probe. Cosmids identified and confirmed to hybridize with the approximately 145-kb *Dra*I fragment were chosen for further characterization (Table 1). Restriction maps were generated for each cosmid, and small fragments from either end of the cosmids were gel-purified using the Qiaex II gel extraction kit (Qiagen, Inc., Valencia, CA, U.S.A.). Overlapping cosmids were identified using these fragments as hybridization probes. In addition, the small fragments were used as hybridization probes against the library in order to identify more cosmids that hybridized with the approximately 145-kb *Dra*I fragment.

Cosmids hybridizing to the approximately 145-kb *Dra*I fragment of *P. syringae* pv. *syringae* strain B301D were not all contiguous, and cosmid walking did not identify new cosmids from the library. DNA fragments from the ends of noncontiguous cosmids were used as hybridization probes against B301D genomic DNA digested with various restriction endonucleases to identify large DNA fragments that span the genomic gap. Large DNA fragments were isolated by gel purification and ligated into pBluescript II SK in order to construct a subgenomic library. This subgenomic library was screened using the DNA fragments from the gap region as a hybridization probe. Positive clones were chosen for further characterization as described above.

Construction of mini-Tn5 disruptions and introduction of these disruptions into the genome of *P. syringae* pv. *syringae* strain BR334 by marker exchange.

Using the restriction map constructed from the overlapping cosmids, fragments chosen for subcloning were located approximately 15 to 20 kb apart along the approximately 145-kb *Dra*I fragment. Fragments were isolated by gel purification from selected cosmids and subcloned into pBluescript II SK. Each of the new subclones was mapped using restriction endonucleases, and unique restriction sites were identified within each subclone. The subclones carried in pBluescript II SK were then digested using the unique restriction endonuclease and polished using T4 DNA polymerase (Gibco-BRL Life Technologies, Inc.) to create blunt ends. The mini-Tn5 cassette (de Lorenzo and Timmis 1994) was digested with *Sma*I, gel-purified, and blunt-end-ligated into the respective subclones to construct a disruption.

tion in the DNA sequence. The DNA flanking the mini-Tn5 cassette were sequenced using primers constructed to prime forward and reverse directions on the mini-Tn5 cassette (forward: 5' GGCTTCGAAATCGTTTTTC 3'; reverse: 5' GCTCACAGCCAACTATC 3'). The inserts from each subclone carrying the mini-Tn5 cassette were then gel-purified and ligated into pBR325. These constructs were introduced by electroporation into competent cells of DH10B for maintenance of the plasmid and into competent *P. syringae* pv. *syringae* cells of strains B301D and BR334 for marker exchange mutagenesis as described by Scholz-Schroeder (2001). Resultant Km^r colonies were screened for loss of the vector and presence of the mini-Tn5 cassette by Southern hybridization (Table 1). Recombinant *P. syringae* pv. *syringae* strains were screened for syringopeptin and syringomycin production using the bioassays described above. Complementation *in trans* for each insertion was performed by introduction of the parental cosmid by electroporation into the mutant strain as described by Scholz-Schroeder (2001).

Construction of the insertional mutagenesis map of the approximately 145-kb *DraI* fragment.

DNA from recombinant *P. syringae* pv. *syringae* strains carrying the mini-Tn5 cassette were analyzed by CHEF-PFGE as described above. The DNA were then transferred to Hybond membrane (Amersham Pharmacia Biotech, Inc.) for Southern analysis. In addition, all *P. syringae* pv. *syringae* mutant strains were analyzed by traditional gel electrophoresis and Southern hybridization for confirmation of the low-molecular-weight fragments. The probe for Southern analysis consisted of a pool of DNA from all the cosmids that hybridized to the approximately 145-kb *DraI* fragment and lambda DNA for hybridization to the molecular weight markers. The size of the Tn3HoHo1 (14.25 kb) is quite large and it was taken into account when constructing the restriction map. We identified one *DraI* site near the end of the Tn3HoHo1 insertion, most likely within the *tnpA* gene. This added approximately 11 kb to the large arm of the *DraI* fragment. It is important to note that the size estimates for the large and the small arms of the *DraI* fragment were made not only with the CHEF-PFGE restriction mapping, but also with the restriction mapping of the cosmids. Using the information together, the size estimates and location of the various insertions were completed.

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