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Sequence Analysis and Phylogeny of VirD4 Protein of Type IV Secretion System in Gram-Negative Bacteria

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Abstract: Sequence analysis and phylogeny of VirD4 protein of Gram-negative bacteria were compared to the classification of the family and knowledge of the evolutionary relationships of its members. Sequences of the gene encoding VirD4 of isolated from *Pseudomonas syringae* pv. *Syringae* were used to sequence searches of members of VirD4 protein and putativeVirD4 protein using Basic Local Alignment Search (BLAST). Sequence analysis revealed high amino acid identity among VirD4 proteins. The central region of VirD4 proteins is generally well conserved in comparison to the terminal regions. The phylogenetic analysis was performed among VirD4 proteins and putativeVirD4 protein in 41 species bacteria. In the phylogenetic tree sequences compared were distributed in several well-defined subfamilies. Each subfamily display relationships that are congruent with other subfamilies.

Key words: VirD4, type IV secretion system, gram-negative bacteria

INTRODUCTION

In gram-negative bacteria, secretion of macromolecules across the two membranes takes place using diverse macromolecular transport assemblies, from simple one-component systems to complex multicomponent machineries. The Type IV Secretion System (T4SS) is one of the five major secretion systems that are capable of exporting virulence factors across the membranes of gram-negative bacteria. T4SSs are defined as macromolecular transfer systems, the components of which are homologous in sequence and probably in structure to those of conjugative transfer systems of naturally occurring plasmids (Ding *et al*., 2003). The T4SS family members have broadly been grouped into three categories; conjugation, effector translocation and DNA uptake and transformation (Ding *et al*., 2003; Shamaei-Tousi *et al*., 2004). More recently, great interest in type IV secretion has resulted from the discovery that agrobacteria use a related system to transfer a segment of their nucleic acid, called T-DNA, together with specific proteins, to plant host cells in a process that results in cancerous transformation (Christie and Vogel, 2000; Cascales and and Christie, 2004). A related export system mediates pertussis toxin secretion and the *Helicobacter pylori* and *Legionella pneumophila* genomes encode genes related to T4SS system constituents that are important for virulence (Ramarao *et al*., 2000; Segal *et al*., 1998).

Type IV secretion system, composed of the VirD4 Type IV Coupling Protein (T4CP) and the VirB mating pair formation (Mpf) proteins. VirD4 proteins are present in all conjugative systems and play a role as coupling proteins, linking DNA-protein substrate to the membrane pore (Yeo and Waksman, 2004; Ding *et al*., 2003; Gomis-Ruth *et al*., 2004). VirD4 proteins thought to mediate interactions between the DNA-processing (relaxosome) and mating pair formation (T4SS) systems (Llosa *et al*., 2002; Schroder and Lanka, 2003).

Cao and Saier (2001) have been reported phylogenetic analysis of virB genes of type IV secretion system in many Gram-negative bacteria, but phylogenetic relationship of VirD4 gene of IV secretion system in Gram-negative bacteria have not previously been defined.

In this study, we have accomplished the analysis sequences VirD4 proteins of Gram-negative bacteria and performed a phylogenetic hypothesis in order to check the relationships between VirD4 in Gram-negative bacteria.

MATERIALS AND METHODS

Isolation and characterization of *P. syringae* pv. *Syringae*: Deciduous stone fruit trees with cankers, bud and blossom blights, leaf spots and shoot diebacks were collected from several commercial nurseries in the Kordestan, August of 2006. Isolation and purification of *P. syringae* pv. *Syringae* has been described

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by Moore *et al*. (2001). Fluorescent, oxidase-negative, gram-negative were further characterized by two determinative schemes: LOPAT tests (Klement, 1963) and GATTa tests (Latorre and Jones, 1979). INA of P. syringae pv. Syringae was determined by the method of Vali as modified by Lindow *et al*. (1978). Cells were suspended to a concentration of approximately 1×108 cfu mL^{-1} (OD590 = 0.3) in Sterile Distilled Water (SDW). Ten drops (10 µL each) of cell suspension were pipetted onto a paraffin coated sheet of aluminum foil floating on a 70% ethanol solution maintained at -5°C (Exacal 300 bath, Neslab Instruments Inc., Portsmouth, NH). The number of drops that froze within 10 min was recorded; strains with eight or more frozen drops were considered INA.

Plasmid DNA extraction and PCR amplification: Plasmid DNA was isolated from *P. syringae* pv. *Syringae* by alkaline lysis (Sambrook *et al*., 1989). Restriction enzyme digests, isolation of DNA fragments and agarose gel electrophoresis, were performed using standard protocols (Sambrook *et al*., 1989).

Two primers were synthesized by the Fazabiotic Company: forward primer, 5' CAGCTACGGGCAACAG-3' and reverse primer, 5'-CACTTCAGACGCTTTGCC-3'. Two primers were designed from the VirD4 gene of *P. syringae* (Gen- Bank Accession No. AY342395.1) to produce a 1443 bp amplicon in PCR amplifications with genomic DNA from *P. syringae* pv. *Syringae*.

PCR amplifications were performed in a total of 25 µL. Reaction mixtures consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.81 M each primer, 200 L mol each dNTP, 1U Ampli*Taq* DNA polymerase and 40 ng of bacterial DNA. The reactions were performed in a acycler termal cycle, Biorad. Denaturation was at 94°C for 4 min for the first cycle and 94°C for 45 sec for each subsequent cycle. Annealing and elongation were at 56°C for 30 sec and 72°C for 54 sec, respectively, for 5 cycles adding 2 sec to the annealing time per cycle. Reactions were terminated after a final 5 min elongation at 72°C. The amplicons in the reaction mix were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide and photographed under UV illumination (Fig. 1). All parts of this study were done at least twice.

DNA sequencing and sequence analysis VirD4 gene: Sequencing of the PCR product in was carried out in an automated DNA sequencer (ABI PRISM 3100, Perkin-Elmer). After translation of VirD4 gene, sequence searches of different members of VirD4 protein of Gram-negative bacteria were carried out using Basic local alignment search (BLAST) (Altschul *et al*., 1997). Clustalw2 was

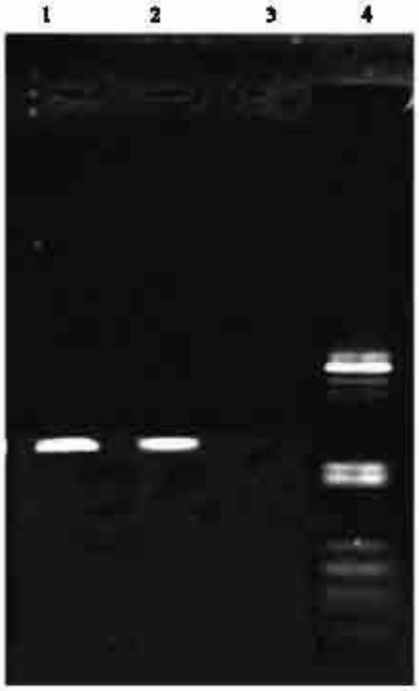

Fig. 1: Ethidium bromide stained agarose (1%) gel electrophoresis of PCR products of VirD4 isolates using specific primers from *P. syringae* pv. *Syringae*. Lanes 1 and 2: PCR products of VirD4. Lane 3: negative control PCR reaction that contained no template DNA. Lane 4: λ-DNA/*Pst*I ladder

used to align all 54 VirD4 protein sequences (http://www.ebi.ac.uk/Tools/clustalw2). Deduced amino acid sequences were further assembled into multiple sequence alignment.

Phylogenetic tree on sequence was also constructed, using neighbor-joining method of MEGA 4.1 (Tamura *et al*., 2007). The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program, was calculated from 10,000 random resamplings.

The organismal abbreviations for organisms that encode VirD4 protein or putative VirD4 protein include: Xax, *Xanthomonas axonopodis*; Eru, *Ehrlichia ruminantium*; Ama, *Anaplasma marginale*; Mpe, *Methylibium petroleiphilum*; Apu, *Aeromonas punctata*; Ret, *Rhizobium etli*; Cje, *Campylobacter jejuni*; Psys, *Pseudomonas syringae*; Btr, *Bartonella tribocorum*; Bpe, *Bordetella petrii*; Rfe, *Rickettsia felis*; Rty, *Rickettsia typhi*; Aph, *Anaplasma phagocytophilum*; Rms, *Rickettsia massiliae*; Pth, *Pelotomaculum thermopropionicum*; Nse, *Neorickettsia sennetsu*; Ech, *Ehrlichia chaffeensis*; Xca,

Xanthomonas campestris; Atu, *Agrobacterium tumefaciens*; Hpy, *Erwinia amylovora*; Lpn, *Legionella pneumophila*; Ots, *Orientia tsutsugamushi*; Lme, *Leuconostoc mesenteroides*; Bvi, *Burkholderia vietnamiensis*; Fea, *Frankia* sp.; Sus, *Streptococcus suis*; Vvu, *Vibrio vulnificus*; Cje, *Campylobacter jejuni*; Swi, *Sphingomonas wittichii*; Bxe, *Burkholderia xenovorans*; Lca, *Lactobacillus casei*; Sal, *Sphingopyxis alaskensis*; Fjo, *Flavobacterium johnsoniae*; Xau, *Xanthobacter autotrophicus*; Bsp, *Bradyrhizobium sp*; Wen, *Wolbachia endosymbiont*; Nwi, *Nitrobacter winogradskyi*; Msp, Mycobacterium sp., Rpa, *Rhodopseudomonas palustris*; Ppe, *Pediococcus pentosaceus*; Bsp, *Bradyrhizobium* sp.

RESULTS AND DISCUSSION

After sequence searches of members of VirD4 protein, we aligned the all VirD4 amino acid sequences. Sequence analysis revealed high amino acid identity Xax VirD4 protein with Xca (93%); Eru with Ech (84%) and Ech (73%); Ama with Aph (75%); Ret with Atu (76); Rfe with Rty (95%) and Rms (97%); Aph with Ech (77%); Ots with Rty (72%) and Rms (72%); Rty with Rms (95%). High sequence identity of VirD4 proteins also reveals that they are well conserved in among bacteria (Table 1). Further, sequence analysis revealed low amino acid identity VirD4 proteins among Lpn, Hpy, Pth, Bpe, Btr, Cje, Apu, Mpe and with another Gram-negative bacteria. High and low identity of VirB of IV secretion system also been reported in Gram-negative bacteria (Cao and Saier, 2001).

The multiple alignment of the members of VirD4 protein revealed several regions of sequence conservation separated by gaps. No fully conserved residues were identified in the members of VirD4 protein multiple alignment. Multiple alignment of amino acid sequences showed many substitutions dispersed all along the length of the protein. However, no unique aa substitutions were observed in the protein sequence.

TrwB, the VirD4 protein, is a protein of 507 residues and binds single-stranded DNA (ssDNA) and doublestranded DNA nonspecifically and independently of nucleoside triphosphate (NTP) binding (Moncalian *et al*., 1999). The crystal structure of a soluble fragment lacking the 70 residues of the N-terminal transmembrane part (TrwBΔN70) unveiled the molecular architecture of TrwB (Gomis-Ruth and Coll, 2001; Gomis-Ruth *et al*., 2002). Three representative states of TrwB?N70 are available to date: protein-substrate, proteinproduct and apoprotein. The TrwBΔN70 monomer has an orange slice shape (Fig. 2A). It consists of two domains: a cytosol-oriented all-α-helical domain (AAD) and a membrane-proximal Nucleotide-Binding Domain (NBD). The NBD is composed of a central, twisted, nine-stranded, mixed β-pleated sheet, which is flanked by four and seven helices on either side. On top of the NBD, the smaller AAD (residues Gly184 to Gly297) is inserted between β 4 and α L of the NBD (Gomis-Ruth *et al*., 2002). This AAD contains seven helices. Topologically, the NBD reveals a high structural similarity to the equivalent domain of RecA and other RecA-like core-encompassing enzymes. Although helicase motifs cannot be found in the sequences of VirD4 proteins, several

Table 1: Deduced amino acid sequence identity among different members of virD4 protein

	Xax	Eru	Ama	Mpe	Apu	Ret	Cje	Psys	Btr	Bpe	Rfe	Rty	Aph	Rms	Pth	Nse	Ech	Xca	Atu	Hpy	Eam	Lpn	Ots
Xax		30	29	27	36	30	20	25	14	6	26	29	29	29	17	29	30	**93**	27	24	24	28	29
Eru	30		**67**	26	32	23	19	25	17	9	55	**62**	**71**	**62**	14	**71**	**84**	29	24	18	20	25	**65**
Ama	29	**67**		27	31	22	18	26	17	8	54	**61**	**75**	**62**	16	**70**	**73**	28	23	16	22	23	**63**
Mpe	27	26	27		30	16	12	20	10	7	22	21	26	21	18	23	24	27	14	16	17	18	20
Apu	36	22	31	30		26	20	28	14	14	30	32	31	31	15	29	32	37	23	17	24	25	30
Ret	30	23	22	16	26		17	19	16	9	22	23	25	23	15	25	24	29	**76**	15	18	21	26
Cje	20	19	18	12	20	17		13	14	11	15	17	17	17	10	18	18	19	15	15	19	18	18
Psys	25	25	26	20	28	19	13		17	8	21	22	25	22	17	26	23	25	19	18	45	22	22
Btr	14	17	17	10	14	16	14	17		14	15	19	16	18	11	16	17	15	12	16	11	15	21
Bpe	6	9	8	7	14	9	11	8	14		9	9	9	9	11	10	9	9	10	10	7	14	9
Rfe	26	55	54	22	30	22	15	21	15	9		**95**	52	**97**	11	54	54	27	23	21	18	26	**67**
Rty	29	**62**	**61**	21	32	23	17	22	19	9	**95**		**60**	**95**	12	**60**	62	27	22	20	20	28	**72**
Aph	29	**71**	**75**	26	31	25	17	25	16	9	52	**60**		**60**	15	**69**	77	29	24	17	20	23	**64**
Rms	29	**62**	**62**	21	31	23	17	22	18	9	**97**	**95**	**60**		12	61	62	27	23	20	20	27	**72**
Pth	17	14	16	18	15	15	10	17	11	11	11	12	15	12		15	14	17	14	8	12	13	10
Nse	29	**71**	**70**	23	29	25	18	26	16	10	54	**60**	**69**	61	15		**70**	29	25	19	21	26	61
Ech	31	**84**	**73**	24	32	24	18	23	17	9	54	**62**	**77**	**62**	14	**70**		30	23	18	20	24	**66**
Xca	**93**	29	28	27	37	29	19	25	15	9	27	27	29	27	17	29	30		27	22	24	29	29
Atu	27	24	23	14	23	**76**	15	19	12	10	23	22	24	23	14	15	23	27		16	20	21	23
Hpy	24	18	16	16	17	15	15	18	16	10	21	20	17	20	8	19	18	22	16		16	14	21
Eam	24	20	22	17	24	18	19	45	11	7	18	20	20	20	12	21	20	24	20	16		20	20
Lpn	28	25	23	18	25	21	18	22	15	14	26	28	23	27	13	26	24	29	21	14	20		29
Ots	29	65	63	20	30	26	18	22	21	9	**67**	**72**	**64**	**72**	10	**61**	**66**	29	23	21	20	29	

Sequence identity was determined by the clustal W2 method. High identity among proteins are shown in bold font

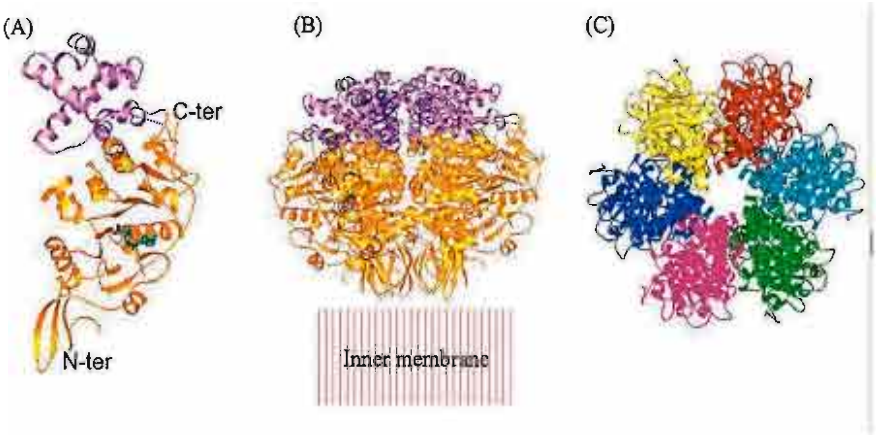

Fig. 2: Molecular features of TrwB, (A) Ribbon presentation of TrwBΔN70 bound to ADP. The N and C termini are indicated (N-ter and C-ter, respectively). Magenta, AAD; gold, NBD. A bound ADP molecule is shown as a cyan stick model to position the nucleotide-binding site, (B) TrwBΔN70 hexamer viewed from the side. The color-coding scheme is the same as that in panel A and (C) TrwBΔN70 hexamer viewed down the small hole at the base of the hexamer formed by the AADs

helicases/ATPase-like proteins also display an AAD besides the core NBD (Gomis-Ruth *et al*., 2002). However, the TrwB AAD appears to bear significant structural similarity only to NTD1 of the site-specific recombinase XerD of the λ-integrase family (Subramanya *et al*., 1997) and thus it has been proposed to contribute to DNA binding (Gomis-Ruth *et al*., 2002). Six TrwBΔN70 subunits associate tightly to form an almost orange-shaped hexamer, somewhat flattened at both poles (Fig. 2B, C).

The strong structural similarity of TrwB to DNA ring helicases suggests that the ssDNA might pass through the central channel of the particle (Guasch *et al*., 2002). Based on this observation, a working model was proposed: ATP binding and hydrolysis could induce a molecular switch mechanism affecting the channel or triggering domain rearrangements and thereby promote DNA binding and displacement through the channel. A main drawback of this model is that the hole at the cytoplasmic side is too narrow for ssDNA to pass through. Also, ATP binding does not appear to be sufficient to trigger conformational changes (Gomis-Ruth *et al*., 2002). Conformational changes may, however, be induced by interaction with relaxosome components or other cellular proteins.

Although similarity-based techniques are essential tools for searching enormous databases and making first approximations of gene homology, phylogeny-based methods offer a more precise and accurate way to classify and name genes (Reeck *et al*., 1987; Thornton and DeSalle, 2000). In this study, using MEGA 4.1 software, we constructed a phylogenetic tree depicting the

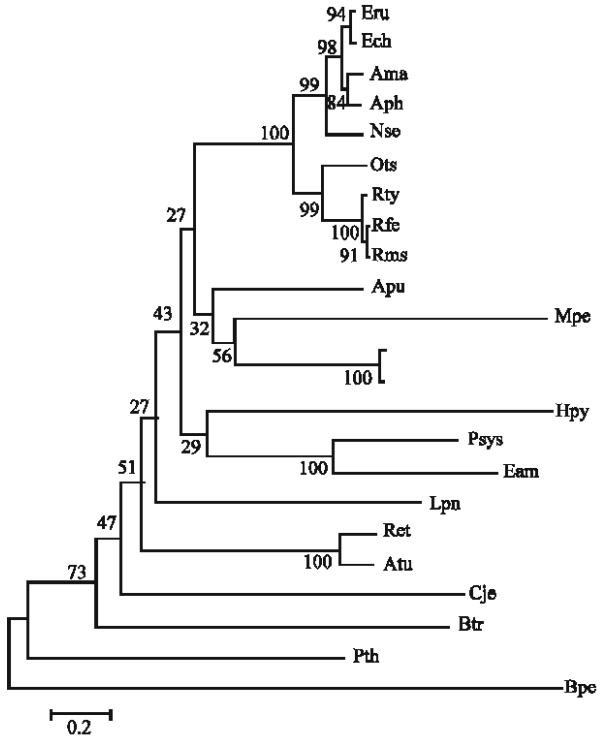

Fig. 3: Phylogenetic analysis of different VirD4 based on amino acid sequence. Un-rooted tree was constructed using neighbor-joining method of MEGA version 4. Numbers on the tree branches represent the bootstrap support calculated per 1000 bootstrap replicates. The scale bar beneath the tree represents the aa substitutions per site

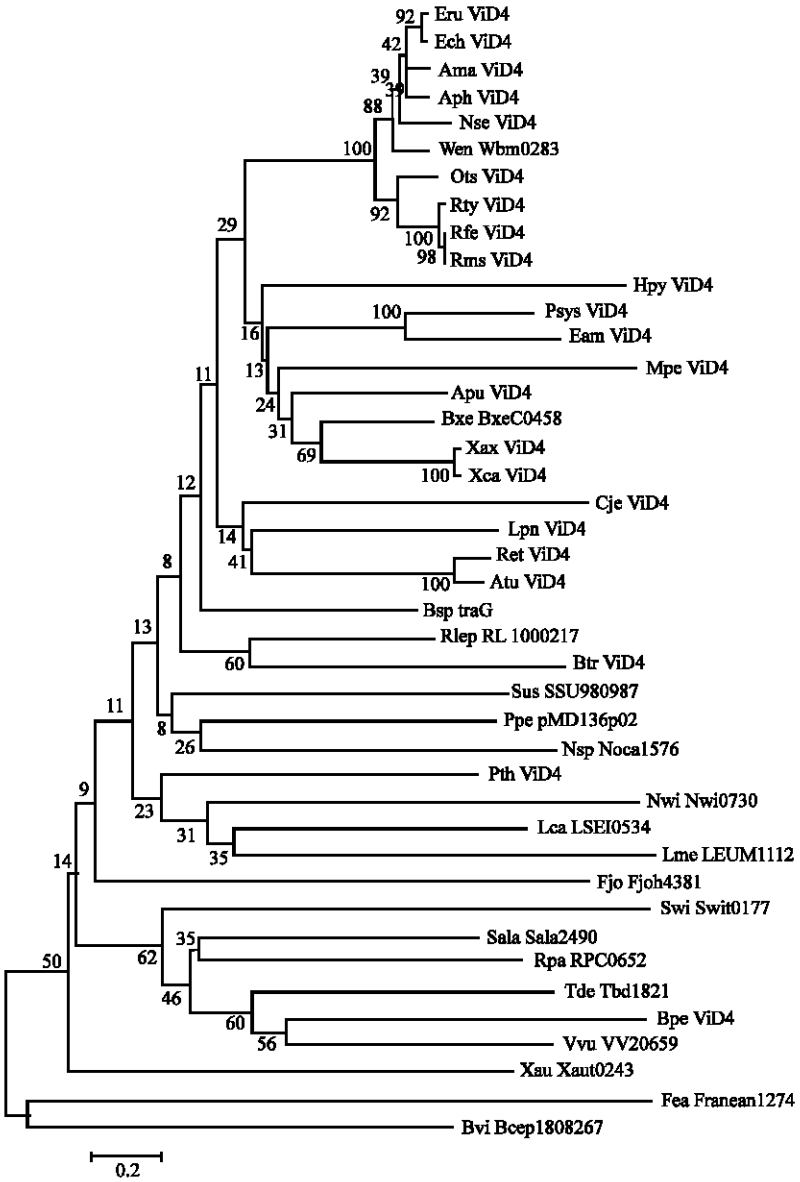

Fig. 4: Phylogenetic analysis of different VirD4 and putative VirD4 proteins based on amino acid sequence. Un-rooted tree was constructed using neighbor-joining method of MEGA version 4. Numbers on the tree branches represent the bootstrap support calculated per 1000 bootstrap replicates. The scale bar beneath the tree represents the aa substitutions per site

relationships of proteinsVirD4 of IV secretion systems. In the phylogenetic tree sequences compared were distributed in several well-defined subfamilies with high bootstrap confidence (Fig. 3). Each subfamily display relationships that are congruent with other subfamilies. The phylogeny presents the following evolutionary scenario. ViD4 protein from Bpe ancestor is all genes VirD4 IV secretion systems that diverged into the several major subfamilies. The phylogenetic tree reveals subfamily of VirD4 proteins family in accordance with expectation assuming that IV secretion systems have evolved without shuffling of constituents between systems.

We examined the evolution of putative VirD4 proteins with VirD4 proteins of IV secretion systems by using phylogenetic analysis (Fig. 4). Phylogenetic analysis shown that Wbm0283 protein from Wen, BxeC0458 from Bxe, RL100217 from Rlep, Nwi0730 from Nwi, LSEI0534 from Lca and VV20659 from Vvu are situated in subfamilies of VirD4 proteins. This phylogenetic analysis

showed a clear distinction among some putative VirD4 proteins and VirD4 proteins. However, the interrelationships among putative VirD4 proteins with VirD4 proteins shown evolutionary scenario these subfamilies.

Functional or structural predictions from primary structure are strengthened when they can take into account the groupings of genes and the history of portions of their sequences (Altschul *et al*., 1997). The sequence analyses and the phylogeny of VirD4 genes as a guide to for detailed molecular genetic analyses and reconstruct functional evolution if we assume that it represents the phylogeny of type IV secretion. In addition to understanding gene relationships, precise classification of genes will benefit other aspects of biological study. However, observations and predictions presented require direct experimental verification. It is hoped that these studies will be forthcoming in the near future.

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