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Identification of potential virulence genes in *Erwinia chrysanthemi* 3937: transposon insertion into plant-upregulated genes

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Abstract *Erwinia chrysanthemi* 3937 is a soft-rotting plant pathogen in Enterobacteriaceae. It attacks a wide range of plant host species. Previously, we identified dozens of *E. chrysanthemi* 3937 genes induced during plant infection by microarray differential display. Here, we have mutated plant-upregulated and putatively plant-upregulated genes in *E. chrysanthemi* 3937 using a transposon insertion method. Of 57 mutants produced, 8 were significantly reduced in maceration in African violet leaves. These 8 *E. chrysanthemi* genes are similar to *Escherichia coli purU* (formyltetrahydrofolate deformylase; ASAP20623) and *wcaJ* (undecaprenylphosphate glucosephosphotransferase; ASAP18556), *Bacillus subtilis dltA* (D-alanine-D-alanyl carrier protein ligase; ASAP19406), *Pseudomonas syringae PSPTO2912* (ABC transporter, periplasmic glutamine-binding protein; ASAP15639), *Pseudomonas aeruginosa pheC* (cyclohexadienyl dehydratase; ASAP19773), *P. syringae syrE* (peptide synthase; ASAP19989), *Vibrio vulnificus VV12303* (unknown protein; ASAP18555), and *Yersinia pestis speD* (*S*-adenosylmethionine decarboxylase; ASAP20536). In some of the genes, possible roles in virulence could be postulated based on the functions of their homologues. This work demonstrated that a low proportion of pathogenicity-related genes were among the plant-upregulated genes of *E. chrysanthemi* 3937. This study and further dissection of these putative virulence genes should lead to new insights into infection mechanisms in pathogens.

Key words *Erwinia chrysanthemi* · *Pectobacterium chrysanthemi* · Transposon insertion · Identification of virulence genes · Plant-upregulated genes

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Introduction

Identification of virulence genes can lead to new insights into infection mechanisms in pathogens and disease-control strategies. One good approach to identify virulence genes is by gene knockout and subsequent evaluation of mutant strains for pathogenicity. In bacteria, classical transposon mutagenesis approaches have often been used for the systematic screening of virulence genes (Hamer et al. 2001). However, some limitations exist in the use of random transposon mutagenesis strategies. Identification and separation of avirulent mutants from a mutant pool are time consuming and often impractical using an in vivo assay. One practical way to screen host-induced bacterial genes is by in vivo expression technologies or differential display of cDNAs including chip technologies (Smith 2000) before mutagenesis. Comparative genomics (Mushegian and Koonin 1996) are also useful to extract pathogen-specific genes as virulence gene candidates. In any event, these primary screenings of host-induced bacterial genes would be important for facilitating identification of virulence genes.

Erwinia chrysanthemi is a soft-rotting plant pathogen in Enterobacteriaceae. It attacks a wide range of plant host species and has been found in other environments such as plant leaf surfaces (Perombelon and Kelman 1980; Haygood et al. 1982), soil (Burr and Schroth 1977; Stanghellini 1982), rivers, and irrigation water (Cother and Gilbert 1990). In *E. chrysanthemi* 3937, pectic enzymes and cellulases have been extensively studied and were found to be important virulence factors in causing the soft-rot symptom (Robert-Baudouy et al. 2000). Iron metabolism (Expert 1999), extracellular polysaccharide (EPS)

(Condemine et al. 1999), and type III secretion pathways (Hueck 1998; López-Solanilla et al. 2001; Yang et al. 2002, 2004; Yap et al. 2005) are also known to be essential for full virulence. In defense against host-derived antibacterial substances or adaptation in host environments, methionine sulfoxide reductase (El Hassouni et al. 1999), flavohaemoglobin (Favey et al. 1995), superoxide dismutase (Santos et al. 2001), and *sap* genes (López-Solanilla et al. 1998) were found to play important roles using mutant strains.

According to the classification by Wassenaar and Gaastra (2001), virulence genes can be divided into three classes. Class I virulence factors include low molecular weight and high molecular weight toxins, enzymes that directly attack the structural integrity of host tissue, and damaging proteins that are directed at or into host cells by various secretion systems. Class II virulence genes that code for factors that regulate expression of true virulence genes (class I genes), activate virulence factors by modification or processing, or are required in other ways for the activity of class I factors. Finally, class III virulence genes encode proteins that adapt pathogens to the host environment. Most of the *E. chrysanthemi* 3937 virulence genes identified so far belong to class I or class II virulence genes.

Recently, we performed microarray profiling of *E. chrysanthemi* 3937 genes that are specifically upregulated or downregulated during plant infection (Okinaka et al. 2002). With prokaryotes, selective gene expression in certain environments generally implies function in that environment. Interestingly, in addition to class I and class II virulence genes, several putative class III genes were revealed to be plant upregulated. Furthermore, most of the plant-upregulated genes have not yet been studied in *E. chrysanthemi* 3937 (Okinaka et al. 2002). Therefore, these plant-upregulated genes are good targets for evaluating possible virulence functions. In this study, we knocked out many of the plant-upregulated or possibly plant-upregulated *E. chrysanthemi* 3937 genes via in vitro transposon insertion and marker exchange and evaluated the virulence of obtained mutants in African violet (*Saintpaulia ionantha*) varieties.

Materials and methods

Gene knockouts in *E. chrysanthemi* 3937

E. chrysanthemi 3937 genes, which were upregulated or putatively upregulated during plant infection (Okinaka et al. 2002), were mutated with an in vitro Tn*5* strategy (EZ::TN ⟨ KAN-2 ⟩ Insertion Kit, Epicentre Technologies, Madison, WI, USA). The pBBR1MCS-4 plasmids, bearing *E. chrysanthemi* 3937 genomic clones with an insertion size of ca. 3 kb used in our previous microarray experiments (Okinaka et al. 2002) were randomly inserted with the transposons. All microarray clones chosen in this study were upregulated in plants and were sequenced. Insertions were physically mapped and precisely located by sequencing from transposon sequence-specific primers (TnF; 5′-ACCTACAACAAAGCTCTCATCAACC-3′, and TnR; 5′-GCAATGTAACATCAGAGATTTTGAG-3′). The plasmid constructs were then electroporated into *E. chrysanthemi* 3937 cells (Yang et al. 2002), and plasmid curing was accomplished by growth of kanamycin-resistant transformants on low-phosphate medium (Ried and Collmer 1987) to select for recombination of the mutation into the chromosome. Mutation in the resultant kanamycin-resistant/ampicillin-sensitive colonies was confirmed by comparing the size of its polymerase chain reaction (PCR) product with the wild-type bacterium using primers flanking the target gene. In addition, a combination of the transposon-sequence-specific primers with primers flanking the target gene was further used to confirm the insertion site for the transposon in each mutant. The P/C ratio (plant/cultural expression rate) of each of the mutated genes is listed in Table 1. The P/C ratio is calculated by dividing the signal intensity of the plant probe with the culture probe in the previous microarray assay (Okinaka et al. 2002). The expression levels of the putative upregulated genes of *E. chrysanthemi* 3937 listed in Table 1 were not determined in previous microarray assays (see Table 1; the P/C ratio is listed as ND, not determined), although the microarray clones containing these putative upregulated genes were found to be upregulated in planta.

Virulence assay

Virulence was tested on African violet vars. Katja and Rosalie leaves by the methods described in Yang et al. (2002). Each mutant was infiltrated into African violet leaves with the wild type infiltrated into the other side of the leaf to eliminate leaf-age-specific variation. After a 40-h post infiltration period, rates of lesion spread by mutant *E. chrysanthemi* 3937 strains were compared with the wild type in at least six individual replicate plants to confirm results. Auxotrophy was determined by a lack of normal cell growth on M9 glucose medium.

Results and discussion

E. chrysanthemi 3937 genes, which were upregulated or possibly upregulated during plant infection (Okinaka et al. 2002), were mutated by transposon insertion (Table 1). In all cases, transposons were inserted into coding regions of the *E. chrysanthemi* 3937 genes. The transposon used was designed to stop translation of mutated genes at insertion sites by providing stop codons in all reading frames and orientations. Fifty-seven mutants were infiltrated into leaves of two African violet varieties, Katja and Rosalie. Bacterial virulence was evaluated by comparing the rates of lesion expansion caused by the mutants with that caused by the wild type (Table 2). In this screening, lesion sizes were compared on each leaf instead of averaging all sizes before comparison. This was necessary because lesion expansion varied with the leaf stage, which would distort the total tendency of virulence if lesion sizes were averaged. Of 57 mutants tested, 8 *E. chrysanthemi* 3937 mutants, TM15639,

Table 1. *Erwinia chrysanthemi* 3937 mutants generated by transposon insertion

ASAP ID	Homologue of the mutated gene (organism)/protein ID[a]	Features of homologous gene	P/C ratio	Original clone[b]
20623	*purU* (*E. coli*)/P37051	Formyltetrahydrofolate deformylase	2.1	ECH1
46527	*fepA* (*S. enterica*)/CAD05061	Ferric enterobactin receptor	2.8	ECH3
19406	*dltA* (*B. subtilis*)/P39581	D-alanine-D-alanyl carrier protein ligase	1.3	ECH5
46916	*VC0200* (*V. cholerae*)/AAF93376	Ferrichrome-iron receptor	1.8	ECH6
18819	*yajO* (*E. coli*)/P77735	NAD(P)H-dependent xylose reductase	2.1	ECH7
20032	*CC2809* (*C. crescentus*)/AAK24773	Peptidase	3.9	ECH10
20033	*MT1758* (*M. tuberculosis*)/P71977	DNA-binding transcriptional regulator	ND	ECH10
18424	*recN* (*E. coli*)/P05824	Recombination and DNA repair	2.6	ECH12
15631	*ydfG* (*E. coli*)/P39831	Putative oxidoreductase	2.6	ECH15
17419	*tsr* (*E. coli*)/P02942	Methyl-accepting chemotaxis protein I	ND	ECH110
18205	*XCC0037* (*X. campestris*)/AAM39356	Pirin-related protein (apoptosis)	ND	ECH111
18206	*nox* (*E. faecalis*)/P37061	NADH oxidase (reduces O_2 - >H_2O)	2.4	ECH111
15124	*perR* (*E. coli*)/Q57083	Peroxide resistance protein (LysR regulator)	5.2	ECH123
20163	*YPO1773* (*Y. pestis*)/CAC90591	Para-aminobenzoate synthase component I	ND	ECH125
20166	*sdaA* (*S. enterica*)/CAD05509	L-Serine deaminase 1	ND	ECH125
15119	*yfeD* (*Y. pestis*)/Q56955	Chelated iron transport	2.4	ECH126
16797	*norV* (*E. coli*)/AAC75752	Putative flavodoxin	5.3	ECH129
16796	*ygbD* (*E. coli*)/AAC75753	Rubredoxin-NAD(+) reductase	ND	ECH129
15639	*PSPTO2912* (*P. syringae*)/AAO56405	ABC transporter, periplasmic glutamine-binding protein	2.6	ECH130
18161	*hcr* (*Y. pestis*)/CAC90188	NADH-dependent HCP reductase	9.8	ECH131
18159	*hcp* (*Y. pestis*)/CAC90189	Prismane	10.4	ECH131
16825	*yceE* (*E. coli*)/P25744	Antiporter, DHA2 family	2.0	ECH139
16461	*lin2949* (*L. innocua*)/CAC98174	Putative transport protein	ND	ECH140
14756	*ydgD* (*E. coli*)/P76176	Protease	4.8	ECH146
14750	*bph1* (*B. bronchiseptica*)/CAE34741	Histone-like protein	42.3	ECH146
16755	*pqqL* (*E. coli*)/AAC74567	Putative peptidase	11.4	ECH202
16754	*yddB* (*E. coli*)/P31827	Unknown protein	ND	ECH202
19989	*syrE* (*P. syringae*)/AAC80285	Peptide synthase 1	ND	ECH205
15511	*cls* (*Y. pestis*)/AAM85599	Cardiolipin synthase (see ECH229 and 234)	1.7	ECH210
19989	*syrE* (*P. syringae*)/AAC80285	Peptide synthase 1	4.5	ECH211
16784	*ptsI* (*Y. pestis*)/CAC92238	PTS system enzyme I component	2.2	ECH217
17546	*yxjG* (*B. subtilis*)/P42318	Unknown protein	4.4	ECH220
17750	*proY* (*Y. pestis*)/AAM84563	Proline permease	ND	ECH220
46892	No matching gene	–	ND	ECH222
19979	*PA5395* (*P. aeruginosa*)/C82971	Unknown protein	6.7	ECH225
174087	*PSPTO1766* (*P. syringae*)/AAO55286	Lipase	2.4	ECH225
16795	*tehB* (*E. coli*)/P25397	Tellurite resistance	4.1	ECH226
19773	*pheC* (*P. aeruginosa*)/Q01209	Cyclohexadienyl dehydratase	3.3	ECH233
19183	No matching gene	–	ND	ECH238
19808	*SCO2861* (*S. coelicolor*)/CAB65591	Unknown protein	ND	ECH238
19805	*yddH* (*E. coli*)/P76121	Unknown protein	ND	ECH238
19975	*BB0826* (*B. bronchiseptica*)/CAE31325	Putative hydrolase	9.1	ECH239
19976	*BB0827* (*B. bronchiseptica*)/CAE31326	Unknown protein	2.6	ECH239
19978	*PP2951* (*P. putida*)/AAN68559	Transcriptional regulator, TetR family	ND	ECH239
18556	*wcaJ* (*E. coli*)/P71241	Undecaprenylphosphate glucosephosphotransferase, polysaccharide biosynthesis	ND	ECH240
18555	*VV12303* (*V. vulnificus*)/AAO10679	Unknown protein	ND	ECH240
18554	*wza* (*E. coli*)/P0A931	Polysaccharide export protein	ND	ECH240
47129	*togB* (*E.chrysanthemi*)/CAC44121	Oligogalacturonide ABC transporter	2.3	ECH242
19103	*BCE3063* (*B. cereus*)/AAS41974	Unknown protein	3.5	ECH309
18568	*ahpF* (*E. coli*)/P35340	Alkyl hydroperoxide reductase large subunit	2.4	ECH316
18570	*ahpC* (*S. typhimurium*)/AAL19559	Alkyl hydroperoxide reductase small subunit	ND	ECH316
19313	*ynbE* (*E. coli*)/NP415900	Unknown protein	ND	ECH323
19314	*acpD* (*E. coli*)/P41407	Acyl carrier protein phosphodiesterase	2.9	ECH323
16787	*cysK* (*S. typhimurium*)/CAD07662	Cysteine synthase A	16.7	ECH404
20536	*speD* (*Y. pestis*)/CAC92642	*S*-Adenosylmethionine decarboxylase	1.9	ECH406
17991	*ybiC* (*E. coli*)/ BAA35467	Dehydrogenase	1.9	ECH503
18401	*ibpB* (*E. coli*)/P29210	Heat shock protein B	3.5	ECH506

P/C ratio, relative gene induction intensity in planta compared with that in culture (Okinaka et al. 2002); ND, not determined

[a] Full-length protein sequences encoded by the mutated genes were searched with BLASTP against all GenBank sequences. Homologues with the best matching scores are listed here. Some homologues are not consistent with our previous list (Okinaka et al. 2002) because not all full-length protein sequences were available in that study

[b] The genes on the listed clones were mutated by in vitro transposon insertion and used for marker-exchange experiments. The clone IDs correspond to those in our previous work (Okinaka et al. 2002)

Table 2. Comparative virulence assays of *Erwinia chrysanthemi* 3937 mutants in two African violet varieties

Mutant ASAP ID	Auxotrophic[a]	Katja			Rosalie		
		W > M[b]	W = M	W < M	W > M	W = M	W < M
Control (Wt)	No	0	13	0	1	17	1
TM20623	Yes	11	4	0	16	0	0
TM46527	No	0	5	1			
TM19406	No	3	2	1	22	4	0
TM46916	No	2	8	0			
TM18819	No	1	3	2			
TM20032	No	0	6	0	1	4	0
TM20033	No	2	4	0	1	5	0
TM18424	No	1	5	0			
TM15631	No	2	8	2			
TM17419	No	2	6	2			
TM18205	No	0	6	0	0	5	0
TM18206	No	1	3	2			
TM15124	No	1	5	0	1	5	0
TM20163	Yes	2	4	0	1	5	0
TM20166	No	1	5	0	1	5	0
TM15119	No	2	5	1			
TM16797	No	2	7	1			
TM16796	No	1	5	0	2	4	0
TM15639	No	17	10	1	13	3	1
TM18161	No	2	4	0	3	8	1
TM18159	No	1	3	2			
TM16825	No	3	5	2			
TM16461	No	2	4	0	3	10	0
TM14756	No	2	1	3			
TM14750	No	5	9	1	11	15	2
TM16755	No	2	4	0	1	5	0
TM16754	No	0	6	2			
TM19989-1	No	18	6	1	17	6	0
TM19989-2	No	3	6	1	6	13	1
TM15511	No	2	4	0	1	5	0
TM16784	Yes	1	5	0			
TM17546	No	1	11	0	1	11	0
TM17750	No	1	11	0	1	11	0
TM46892	No	4	8	0	2	5	1
TM19979	No	2	4	2			
TM174087	No	0	5	1			
TM16795	No	3	9	0	6	10	1
TM19773	No	6	3	0	17	2	0
TM19183	No	2	4	0	1	4	1
TM19808	No	1	5	0	1	5	0
TM19805	No	0	6	0	0	5	1
TM19975	No	1	5	0			
TM19976	No	1	3	2			
TM19978	No	0	4	0			
TM18556	No	5	7	0	15	2	0
TM18555	No	3	7	2	15	9	0
TM18554	No	0	6	0	0	6	0
TM47129	No	0	6	0			
TM19103	No	1	4	1	1	5	0
TM18568	No	2	3	1			
TM18570	No	2	4	0	1	4	1
TM19313	No	2	5	1			
TM19314	No	1	4	1			
TM16787	Yes	1	5	0	0	6	0
TM20536	No	10	4	0	14	1	0
TM17991	No	2	4	0	4	10	2
TM18401	No	1	5	0			

African violet leaves were infiltrated with wild type on one side and the mutant on the other side of the same leaf. Lesion diameters obtained with a mutant were compared with those obtained with wild-type cells on each leaf

[a] Auxotrophy of mutants was tested on M9 glucose plates. Mutants with significantly slow or no growth on the plates were considered as auxotrophic

[b] Number of leaves with smaller, similar, or larger lesions by a mutant compared with the wild type is listed as W > M, W = M, or W < M, respectively. Diameter difference within 25% was considered as not significant and classified as W = M

TM18555, TM18556, TM19406, TM19773, TM19989-1, TM20536, and TM20623 had reduced leaf maceration ability. These 8 *E. chrysanthemi* genes are similar to *Escherichia coli purU* (formyltetrahydrofolate deformylase; ASAP20623) and *wcaJ* (undecaprenylphosphate glucosephosphotransferase; ASAP18556), *Bacillus subtilis dltA* (D-alanine-D-alanyl carrier protein ligase; ASAP19406), *Pseudomonas syringae PSPTO2912* (ABC transporter, periplasmic glutamine-binding protein; ASAP15639), *Pseudomonas aeruginosa pheC* (cyclohexadienyl dehydratase; ASAP19773), *P. syringae syrE* (peptide synthase; ASAP19989), *Vibrio vulnificus VV12303* (unknown protein; ASAP18555), and *Yersinia pestis speD* (*S*-adenosylmethionine decarboxylase; ASAP20536) (Table 2). TM18555 and TM18556 are in the same operon. The reduction of virulence in TM19773, TM19989, and TM20623 reported here was also observed in our previous study (Okinaka et al. 2002). The relative gene induction rates in planta (P/C ratio in Table 1) of these 8 genes were 1.9 to 3.3, which are not very high among the plant-upregulated genes. On the contrary, mutation of homologues of *Y. pestis hcr* and *hcp* (ASAP18161 and ASAP18159), *E. coli pqqL* (ASAP16755), *Salmonella typhimurium cysK* (ASAP16787), and *Bordetella bronchiseptica BB0826* (ASAP19975) were not significantly reduced in virulence, even though the P/C rates of these homologues were high (Table 2). In general, a greater reduction in virulence was observed in Rosalie than in Katja (Table 2). Clear results were obtained for mutants TM18556 and TM18555. In the first case, smaller diameter lesions were observed on the Rosalie leaves than on the Katja leaves. These results indicate that Rosalie is a more stringent host for *E. chrysanthemi* 3937 than Katja. A similar tendency was observed for *hrpG* and *hrpN* mutants in our previous study (Yang et al. 2002). We further compared the diameter of the macerated area on Rosalie leaves caused by wild-type *E. chrysanthemi* 3937 with each of the mutants: TM15639, TM18555, TM18556, TM19406, TM19773, TM19989-1, TM20536, and TM20623. Leaf maceration was significantly reduced in these 8 mutants according to paired-sample *t*-test at $P < 0.05$ (Table 3).

Table 3. Local lesion maceration by wild-type *Erwinia chrysanthemi* 3937 and mutants on African violet variety Rosalie

Bacterial strains	Diameter of lesion (mm)[a]
WT Ech3937	18.78A
TM20623	10.66B
WT Ech3937	15.39A
TM19406	6.25B
WT Ech3937	16.35A
TM15639	10.06B
WT Ech3937	17.30A
TM19989-1	10.41B
WT Ech3937	17.24A
TM19773	8.45B
WT Ech3937	11.91A
TM18556	5.15B
WT Ech3937	16.88A
TM18555	10.67B
WT Ech3937	15.77A
TM20536	5.47B

[a] The diameter of lesions was measured on individual leaves and averaged for at least six individual replicate plants. Means followed by characters, A or B, in each treatment set differed at $P < 0.05$ according to a paired-sample *t*-test

Of these eight mutants with reduced maceration, the *purU* (ASAP20623) of *E. chrysanthemi* 3937 was located downstream of *ychJ* (ASAP20621) (Fig. 1A). The Neural Network for Promoter Prediction (NNPP) program (Reese et al. 1995) located a putative promoter sequence with a high matching score of 0.93 just upstream of the ASAP20621. Thus, ASAP20623 and ASAP20621 are probably cotranscribed from the putative ASAP20621 promoter in an operon. Based on bacterial growth in M9 glucose minimal medium broth, the TM20623 mutant was revealed to be auxotrophic (Table 2). Instead of a true reduction in virulence in the TM20623 mutant, the auxotrophic phenotype may have contributed to the reduced maceration of the *purU* mutant on the host leaves.

The putative virulence gene, ASAP19406, was homologous to the *B. subtilis* D-alanine-D-alanyl carrier protein ligase gene, *dltA*. This candidate gene was linked to another *dltA* homologue (ASAP19407) (Fig. 1B). The two *dltA* homologues overlapped by 94 bp. The downstream gene, ASAP19406, was separated from ASAP19405, the *Clostridium acetobutylicum CAC2192* homologue, by a 98-bp noncoding region. In this 98-bp noncoding region, no promoter sequences were predicted by the NNPP program, although the 98-bp section appeared large enough to accommodate a promoter. Because a putative promoter sequence with a good NNPP score (0.86) was located between ASAP19405 and ASAP19403, the *Acinetobacter ORF4* homologues, these two genes are probably transcribed separately. Taken together, ASAP19407, ASAP19406, and ASAP19405 seemed to be cotranscribed in the operon. Thus, the transposon insertion in the ASAP19406 might have a polar effect on the ASAP19405. In addition, both of the *dltA* homologues, ASAP19407 and ASAP19406, could have similar virulence functions because of the structural similarity in their encoded proteins. In Gram-positive bacteria, *dltA* helps covalent binding of D-alanine to teichoic acids in the cell wall. The positive charge of teichoic acids given by D-alanine binding eventually confers resistance to cationic antimicrobial peptides (Peschel et al. 1999). A similar observation was made with a Gram-negative bacterium, *E. coli.* Bacterial resistance to the cationic peptide, polymyxin B, was caused by reduction of the anionic nature of lipopolysaccharide in the outer membrane (Nummila et al. 1995). These suggest that the increase in the positive charge of bacterial surfaces may be a prevalent strategy among microorganisms to protect themselves from bacteriocin-like molecules that inhibit the growth of competing strains. Several cationic peptides of plant (e.g., defensins and thionins) have been reported to have antimicrobial activity (Hancock and Lehrer 1996; Marshall and Arenas 2003). To resist these cationic peptides, one possible role of gene ASAP19406 (Fig. 1B) is to elevate the positive charge of *E. chrysanthemi* 3937 cell surfaces through their putative function in attaching D-alanine to polysaccharides.

The putative virulence gene in ASAP15639 probably encodes a periplasmic glutamine-binding protein in a

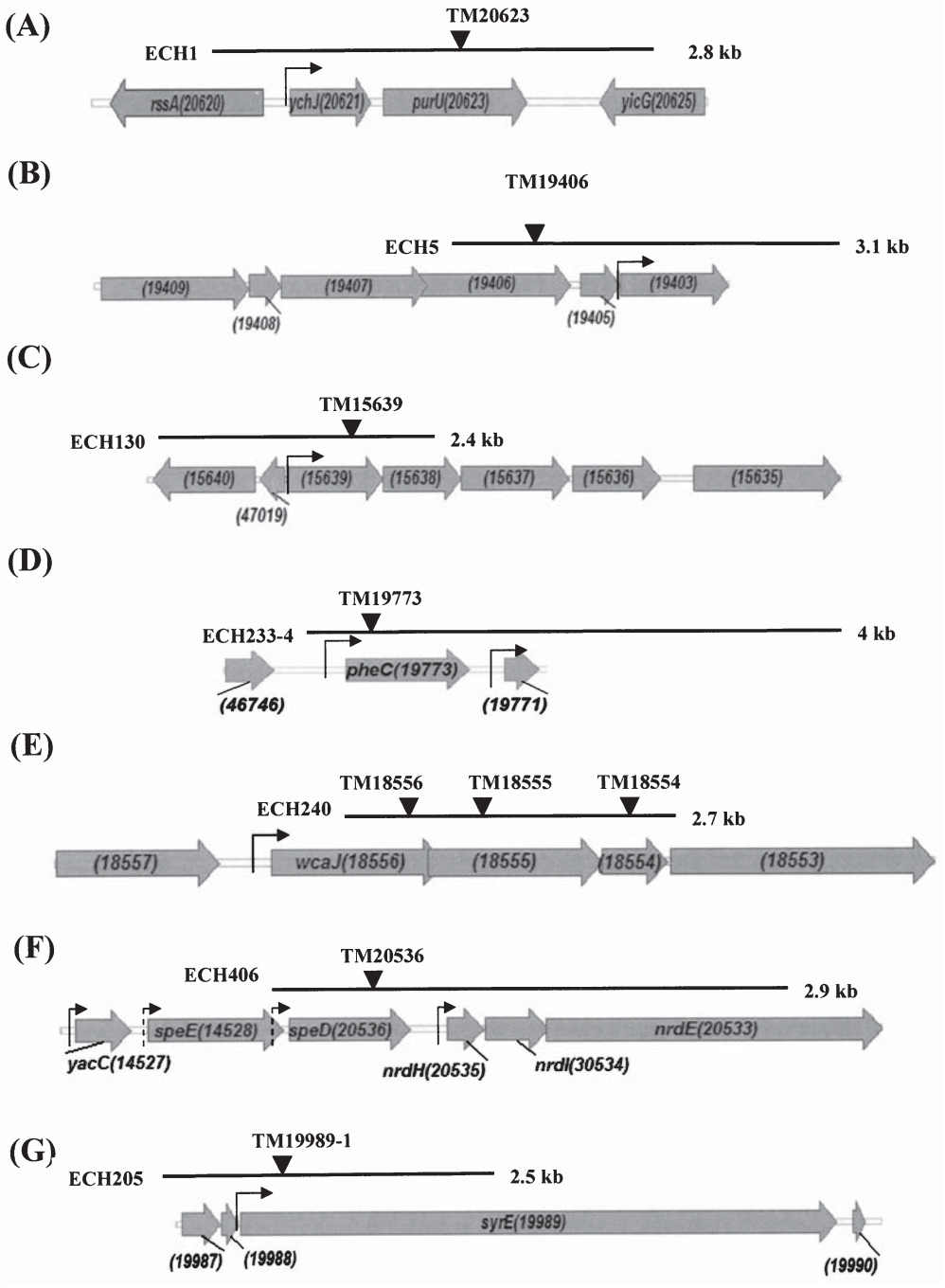

Fig. 1A–G. Mapping of the transposon insertions in the *Erwinia chrysanthemi* 3937 genome. Transposon insertion positions (*filled triangles*) are indicated on the original array clones (*solid bars*) and the open reading frame (ORF) maps. The ASAP IDs of each ORF in *E. chrysanthemi* 3937 genome database and their transcriptional orientations are shown by *open arrows*. The approximate locations of potential or possible promoter sequences detected by the Neural Network for Promoter Prediction program (Reese et al. 1995) are shown by *solid-line arrows* and *broken-line arrows*, respectively

glutamine ABC transporter. The stop codon of ASAP15639 was 8 bp upstream from the ATG start codon of the ASAP15638 (Fig. 1C). This indicates that the ASAP15638 is cotranscribed in an operon with the ASAP15639. Similarly, a small noncoding sequence was found between ASAP15637 and ASAP15636. In addition, there was a 3-bp overlap between ASAP15638 and ASAP15637. Thus, collectively, the four genes from ASAP15639 to ASAP15636 may be cotranscribed, and the transposon insertion in ASAP15639 could have a polar effect on the other three genes. In group B streptococci, mutants for the glutamine transport gene, *glnQ*, had decreased fibronectin adherence and virulence as well as lower cytoplasmic glutamine (Tamura et al. 2002). In this case, the lowered level of cytoplasmic glutamine may negatively regulate fibronectin gene expression. Numerous studies have demonstrated that gene expression may be regulated through alterations in cytoplasmic amino acid levels (Jayakumar et al. 1987; Bender and Magasanik 1977; Fink et al. 1999). Therefore, the disruption of the ASAP15639 mutant might affect virulence-related gene expression through reduced cytoplasmic glutamine. It cannot be ignored that ASAP15639 itself has a virulence function in *E. chrysanthemi* 3937.

Another virulence gene candidate, the *P. aeruginosa pheC* homologue (ASAP19773), seemed to be comprised of a monocistronic operon (Fig. 1D) because the candidate

gene was isolated from large intergenic regions (501 and 244 bp) with putative promoter sequences having high NNPP scores (0.99 and 1.0, respectively). No difference in bacterial growth was observed between wild-type *E. chrysanthemi* 3937 and TM19773 mutant in minimal medium broth. Therefore, virulence reduction in the TM19773 mutant probably originated from the disruption of the *pheC* function. The PheC protein mediates the phenylalanine biosynthetic pathway, converting prephenate to phenylpyruvate. Several phenylpropanoid phytoalexins, e.g., coniferyl alcohol, are produced from phenylalanine through the shikimic acid pathway (Cao et al. 2001). Perhaps such bacterial phenylalanine synthesis pathway proteins consume precursors for phenylalanine biosynthesis in host plants. Phenylalanine production in hosts is thus reduced, consequently reducing production of plant defense compounds such as phytoalexins (Hammerschmidt 1999).

The putative virulence genes ASAP18556 and ASAP18555 are capsular polysaccharide biosynthesis proteins (Fig. 1E). A 134-bp intergenic space with a potential promoter sequence (NNPP score = 1.0) is located between ASAP18557 and ASAP18556. In addition, ASAP18556 and the next eight genes were aligned without possible promoter sequences (data not shown). The genes located downstream from ASAP18556 were related to polysaccharide synthesis. Because virulence was not reduced in the ASAP18554 mutant (Table 2), insertion disruption of ASAP18556 or ASAP18555 did not have a polar effect on the ASAP18554 or the adjacent putative cistrons. Furthermore, virulence was reduced more in ASAP18556 than in ASAP18555. Therefore, the ASAP18556 mutant phenotype was likely affected by a polar effect on ASAP18555 as well as by the defect in ASAP18556 itself. An extracellular capsule is generally present on the outer membranes of Enterobacteriaceae and is important for bacterial biofilm formation and resistance to desiccation (Ophir and Gutnick 1994). It also protects against environmental stress from acid and heat (Mao et al. 2001). In *Ralstonia solanacearum*, both lipopolysaccharides and exopolysaccharides were important virulence factors for pathogenesis in plant hosts (Cao et al. 2001).

The ASAP20536 (*Y. pestis speD* homologue) seemed to be comprised of a monocistronic operon or the last cistron of a putative *speED* operon (Fig. 1F) because a 216-bp noncoding spacer, which contained a potential promoter sequence (NNPP score = 0.92), was located between ASAP20536 and ASAP20535. This prediction is supported by our previous report (Okinaka et al. 2002) that ASAP20533 (*nrdE*) was plant downregulated in contrast to the plant upregulation of ASAP20536. Therefore, the insertion disruption of the ASAP20536 probably affects spermidine synthesis in *E. chrysanthemi.* Transposon insertion into the ASAP20536 reduced virulence in *E. chrysanthemi* (Table 2). In *E. coli*, mutants completely deficient in spermidine biosynthesis still grew at a near-normal rate (75%–85% of the wild-type growth) in a minimal medium without spermidine (Tabor et al. 1978; Xie et al. 1993). Compared with wild-type *E. chrysanthemi* 3937, bacterial growth was not reduced in the TM20536 mutant. Even though TM20536 is a true virulence gene in *E. chrysanthemi*, the mechanism by which spermidine or *speD* gene supports bacterial infection is still unknown. Thus far, no evidence has been reported for the involvement of spermidine in bacterial virulence.

Transposon insertion of TM19989-1, approximately 150 bp downstream from the ATG start codon of ASAP19989 (*syrE* homologue), strongly reduced bacterial virulence in African violets (Table 2, Fig. 1G). However, only a slight virulence reduction was obtained for TM19989-2. The transposon insertion of TM19989-2 was located at the C terminal of the *SyrE* homologue. The function of the *SyrE* homologue gene might not be fully disrupted by this transposon insertion. *P. syringae syrE* encodes a component of syringomycin synthase complex. Syringomycins are cyclic lipodepsipeptide toxins with phytotoxic activity and a wide spectrum of antimicrobial and antifungal properties (Guenzi et al. 1998). The *SyrE* homologue protein in *E. chrysanthemi* 3937 was also structurally similar to bacterial antibiotic peptide synthases, such as tyrocidine in *Bacillus brevis* (Mootz and Marahiel 1997), gramicidin S in *B. brevis* (Saito et al. 1994), and bacitracin in *Bacillus licheniformis* (Konz et al. 1997) according to BLASTP searches (http://www.ncbi.nlm.nih.gov/BLAST/) (unpublished data). These data suggest that the *syrE* homologue plays a role in producing a biologically active peptide, which probably kills or weakens host plant cells or competitive microbes.

We did not observe virulence reduction in the *togB* mutant (ASAP47129) (Table 2). In a previous study, *togB* was addressed as a virulence gene, although the reduction in virulence was slight (Hugouvieux-Cotte-Pattat et al. 2001). This discrepancy might occur because of the difference in the virulence assay systems. Hugouvieux-Cotte-Pattat et al. (2001) used detached chicory leaves for the bacterial inoculation assay. A virulence gene for one plant species may not necessarily confer a significant virulence phenotype in another species, although *E. chrysanthemi* has a broad host range.

Aside from *purU* (ASAP20623), seven putative virulence gene candidates were identified from the 57 plant-upregulated or putative plant-upregulated genes screened (Table 2). This efficiency looks higher than that in random transposon mutagenesis experiments (Hamer et al. 2001). However, the efficiency in this study was somewhat lower than expected. One possible reason is the existence of genes with redundant functions. Simultaneous disruptions of such genes would be required to uncover their virulence defects. Another possible reason is that a significant portion of the bacterial genes that are upregulated in plant hosts may not be related to pathogenicity. Both a local maceration assay and a systemic invasion assay are often performed for virulence tests in *E. chrysanthemi* (Condemine et al. 1999; Yang et al. 2004). A systemic invasion assay would be useful in reevaluating the mutants with slightly attenuated virulence (e.g., TM16461, TM14750, TM19989-2, and TM16795) in future studies.

Our results indicate that some potential class III genes are important for this necrotrophic bacterium to infect host

plants. Further evaluation of potential class III virulence genes as well as class I and class II genes should aid our understanding of the virulence mechanisms in *E. chrysanthemi* even though the evaluation is relatively difficult and labor intensive. The possible roles of these putative virulence genes are discussed here according to the functions of their structural homologues identified with BLASTP searches. Thus, evaluation of the in vitro activity of the candidate gene products is necessary to provide more concrete evidence for their functions. Furthermore, determination of transcriptional units including the candidate genes would probably provide a better understanding of the roles of the genes in pathogenicity. Verification that mutant phenotypes may not be derived from any undesirable spontaneous mutations by gene complementation assays should also be undertaken.

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