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Microarray Profiling of *Erwinia chrysanthemi* 3937 Genes That are Regulated During Plant Infection

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Microarray technology was used to identify genes in *Erwinia chrysanthemi* 3937 that are specifically up- or down-regulated in a plant host compared with growth in laboratory culture medium. Several genes were plant down-regulated, and almost all of them were homologues of well-known housekeeping genes, such as those encoding metabolic functions, oxidative phosphorylation components, and transcription or translation processes. On the other hand, almost all of the plant up-regulated genes were involved with specialized functions, including already known or new putative virulence factors, anaerobiosis, iron uptake, transporters or permeases, xenobiotic resistance, chemotaxis, and stress responses to reactive oxygen species and heat. A substantial number of the plant up-regulated genes do not appear to be directly involved in damaging the host, but are probably important in adapting the pathogen to the host environment. We constructed insertion mutations in several of the plant up-regulated *E. chrysanthemi* 3937 genes. Among these, mutations of *Bacillus subtilis pps1*, *Escherichia coli purU*, and *Pseudomonas aeruginosa pheC* homologues reduced virulence on African violet leaves. Thus, new insights were obtained into genes important in bacterial virulence.

Many pathogens of animals and plants survive in unique alternative habitats in which they are likely to employ distinct sets of expressed genes. A current approach to identifying gene sets expressed by organisms in alternative growth environments is microarray gene expression technology (Danchin and Sekowska 2000; DeLisa et al. 2001; Wei et al. 2001). Genes of interest are arrayed as DNA microspots on glass slides or membranes and the arrays are hybridized to labeled cDNAs produced from mRNA of the target organism grown in particular alternative environments. The major advantage of the approach is that, unlike conventional reporter gene or hybridization strategies, microarrays can simultaneously produce relative expression data for thousands of genes in the target organism, and no prior knowledge of the genes or their regulation is required. Microarrays, therefore, represent an appealing approach to identifying bacterial gene sets that are up-regulated by growth in specific environments, and are particularly attractive for studying gene expression by pathogenic bacteria in

their normal hosts. We report one of the first studies in which microarrays have been used to identify genes that are specifically up-regulated by bacteria in the pathogenic phase compared with growth on laboratory culture media.

Erwinia chrysanthemi 3937 is a soft-rotting plant pathogen in the *Enterobacteriaceae* family. It attacks a wide range of plant host species and also has been reported to exist in alternative environments such as plant leaf surfaces (Haygood et al. 1982; Perombelon and Kelman 1980), soil (Burr and Schroth 1977; Stanghellini 1982), and rivers and irrigation water (Cother and Gilbert 1990). Accordingly, we have established the goal of utilizing microarray gene expression as a tool to identify gene sets that are specifically expressed by the bacterium in these alternative environments. With prokaryotes, selective gene expression in a certain environment generally implies function in that environment. In this article, we describe initial results defining the *E. chrysanthemi* 3937 gene set expressed during the pathogenic phase in African violet leaves.
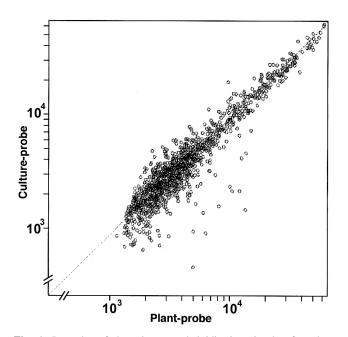
RESULTS

E. chrysanthemi 3937 genomic arrays with approximately 5,000 randomly selected 3-kb clones were prepared. The arrays were hybridized with Cy3-labeled cDNA probes prepared with total RNA from plant-grown bacteria or those cultured in Luria-Bertani (LB) medium (plant-probe or culture-probe, respectively). In each array clone, normalized signal intensity obtained with plant-probe was divided by that with culture-probe to calculate the plant/culture expression rate (P/C ratio). Based on average P/C ratios obtained from replicated hybridization experiments, down-regulated clones (P/C $\leq$ 0.66) and up-regulated clones (P/C $\geq$ 1.5) were selected as statistically significant with some exceptions. Of the 5,000 *E. chrysanthemi* 3937 clones analyzed, 74 plant up-regulated clones and 15 down-regulated clones were identified (Fig. 1, Tables 1 and 2). Several plant up- and down-regulated clones were end sequenced and the resulting data were analyzed by BLASTX, BLASTP, and BLASTN searches. Many clone inserts were completely sequenced by sequential primer walking. Clones generally contained two to four open reading frames (ORFs) (Tables 1 and 2), only a few of which had previously been seen in *E. chrysanthemi* 3937 (*argG* and *pecS* [ECH228], *pelDE* [ECH408], *rssA* [ECH1], *estV* [ECH223 and ECH225], *togB, kdgM,* and *paeX* [ECH242]) or the related pathogen *E. carotovora* (*prt* [ECH235], *rsmC* [ECH6], and phage invertase [ECH397]) (Chatterjee et al. 2000).

Excepting cases where only single genes occurred in particular clones, we could not definitively assign ORFs that were being up- or down-regulated in the plant until individual ORFs were amplified by polymerase chain reaction (PCR) and rear-

rayed. Therefore, we amplified 59 interesting ORFs in the plant up-regulated clones by PCR, spotted them onto slide arrays, and performed hybridization experiments to identify plant up-regulated ORFs (Table 2, column 3). In these ORF hybridization experiments, P/C ratios higher than those from the parental 3-kb clone (Table 2, column 2) were often obtained. However, some plant up-regulated 3-kb clones missed such strongly expressed ORFs (e.g., ECH11 and ECH503). One reason may be the use of concentrated DNA and low hybridization temperature (see below) with which nonspecific binding was higher than with the original conditions (data not shown). Nevertheless, 48 plant up-regulated ORFs were confirmed by the re-array hybridizations.

Relatively few plant down-regulated clones were observed compared with plant up-regulated clones, and these generally contained housekeeping genes (Table 1). Most of them were related to the TCA cycle/oxidative phosphorylation (ECH109, ECH117, and ECH409), protein synthesis (ECH212, ECH317, ECH327, ECH407, and ECH411), or transcription (ECH 302, ECH312, and ECH410). These results may reflect a general down-regulation of aerobic metabolism and bacterial growth rate in the plant compared to the rich LB culture medium.

Plant up-regulated genes were more numerous in the array experiments (Fig. 1, Table 2), and these were generally not housekeeping genes. Although new putative polygalacturonase (ECH14), protease/peptidase (ECH10, ECH11, ECH146, ECH202, ECH314, and ECH405), and peptide synthase (ECH5, ECH16, ECH205, and ECH211) virulence factors were identified as plant up-regulated, the microarray screening has thus far surprisingly not encountered type III secretion (*hrp*) genes, known to secrete putative virulence factors in *E. chrysanthemi* (López-Solanilla et al. 2001; Yang et al. 2002). This despite the fact that the *E. chrysanthemi* 3937 *hrpG* and *hrpN* genes, arrayed as controls, showed the expected plant up-regulation (Table 2). The *rsmC* global virulence regulator of *E. carotovora* (Cui et al. 1999) was observed for the first time in *E. chrysanthemi* on plant up-regulated clone ECH6, and may indicate that the *rsm* system functions in *E. chrysanthemi*.

Fig. 1. Log plot of the microarray hybridization signals of random *Erwinia chrysanthemi* genomic clones obtained using culture- and plant-probes. The signal intensity obtained with plant-probe (*x* axis) was plotted against that with culture-probe (*y* axis) after signal normalization. These data were derived from a single hybridization experiment.

We failed to encounter iron-uptake genes previously sequenced from *E. chrysanthemi* (Expert 1999), but observed several new putative iron uptake genes. These included homologues of *Escherichia coli fhuA* (ECH6) and *feoB* (ECH11), *Pseudomonas aeruginosa pfeA* (ECH3), and *yfeABCD* homologues from *Yersinia pestis* (ECH123 and ECH126). The plant up-regulated *E. coli entA* homologue (ECH142) is probably a component of the partially characterized *cbs* operon in *Erwinia chrysanthemi* that encodes production of the virulence-related siderophore, chrysobactin (Franza and Expert 1991).

Four different plant up-regulated clones (ECH5, ECH16, ECH205, and ECH211) showed homology to peptide synthases, known as virulence factors in other bacteria (Gross 1991) but not seen previously in *E. chrysanthemi*. Several putative xenobiotic resistance genes were identified, including a *P. aeruginosa xenB* homologue (ECH142, ECH209, and ECH230) and a gene with weak homology to an oxalate/formate antiporter gene (ECH241). Two tellurite resistance gene homologues were plant up-regulated (an *Escherichia coli tehB* homologue in ECH226 and a *cysK* homologue in clone ECH404) (Vásquez et al. 2001). Any physiological role for these genes in virulence is not yet clear. Three phosphotransferase (PTS) system genes, the PtsI and glucose IIA (ECH217), and glucose IIBC (ECH503) components also were identified. These genes will be interesting to study further in view of the well-known role of the PTS system as a global gene regulator in other bacteria (Saier and Reizer 1994). Homologues of the *E. coli* chemotaxis genes *tsr* (ECH110) and *tar* (ECH222), and the *rstA* and *rstB* putative osmolarity sensors (ECH146, ECH308, ECH314, and ECH405) were present on plant up-regulated clones. Putative oxidoreductases, heat shock, and stress protein genes also were identified (ECH7, ECH15, ECH111, ECH123, ECH147, ECH316, ECH406, ECH503, and ECH506), including homologues of the *E. coli* PerR peroxide resistance regulator protein, the AhpFC alkyl hydroperoxide reductase, and the SpeD S-adenosylmethionine decarboxylase that generates polyamines.

Several recombination/DNA repair genes (homologues of DNA inversion genes [ECH9, ECH229, and ECH307] and *E. coli ssb* [ECH238], *recN* [ECH12], *recF* [ECH237], *gyrB* [ECH237], and *mutL* [ECH13]) also were plant up-regulated, perhaps an indicator of bacterial stress in the plant environment. Induction of transposase genes (ECH142, 209, 230, and 243) also may be a consequence of bacterial stress in the plant. One clone (ECH110) contained a partial ORF with high homology to the microcin self-immunity protein genes of *E. coli* and *Vibrio cholerae*. This raises the possibility that *Erwinia chrysanthemi* may deploy a microcin-similar antibiotic system in host plants (Gonzalez-Pastor et al. 1995). A highly plant up-regulated unknown gene (ECH146, ECH308, ECH314, and ECH405) with low homology to the *Chlamydia hct2* histone-like gene (Brickman et al. 1993) encoded an unusual lysine-rich protein with seven tandem near-perfect 15-amino-acid repeats (PAQKAQAAKKVAKKK). Finally, homologues to two different cardiolipin synthase genes were identified on clones ECH210, 229, and 234. These are of interest because they may imply specific alterations to phospholipid components of the bacterial membrane during growth in a plant host.

Several homologues of *Escherichia coli* genes showed different linkages in plant up-regulated *Erwinia chrysanthemi* clones (e.g., *pepT* and *feoB* in ECH11). This probably means that the *E. chrysanthemi* genome will show the extensive mosaic of gene rearrangements relative to *Escherichia coli* K-12 observed in comparison with *E. coli* O157:H7 (Perna et al. 2001).

We utilized an in vitro Tn*5* insertion strategy to rapidly construct mutations in several ORFs identified as plant up-regulated (Table 2, column 3). Although the number of ORFs thus far mutated is not large, we identified 3 mutants out of 17

screened that showed clear virulence defects on African violet leaves compared with the wild-type bacteria. A mutation in the *Bacillus subtilis* peptide synthase homologue on ECH205 showed reduced virulence, although an insertion in the *P. syringae* peptide synthase homologue of ECH211 did not lead to a reduction in virulence (Fig. 2A). These results may indicate that a cyclic peptide contributes to virulence in *Erwinia chrysanthemi*, although no previous reports exist. Gene knockouts in the plant up-regulated *Escherichia coli purU* homologue on ECH1 and the *P. aeruginosa pheC* homologue on ECH233 (Fig. 2B and C) also markedly reduced bacterial virulence of African violet leaves. The biochemical basis for these virulence defects is not known.

DISCUSSION

The microarray project identified many interesting genes in *Erwinia chrysanthemi* 3937 that are plant up-regulated relative to growth on LB culture medium (Table 3). It was satisfying to see that plant up-regulated clones generally did not carry housekeeping genes or, if they did, that other gene homologues were present on the same clones that might conceivably contribute to virulence. This implies that the microarray project reliably identified plant up-regulated bacterial genes that may contribute to growth and survival in the plant environment. Two major surprises in the project were the relatively small number of *E. chrysanthemi* plant up-regulated clones carrying known virulence genes and the large number of plant up-regulated genes with homology to known DNA repair and recombination, stress protec-

tion, oxidoreductase, xenobiotic degradation, and transporter genes. Accordingly, it is appealing to speculate that many of these latter gene products may favor bacterial growth and development in the plant host environment.

Wassenaar and Gaastra (2001) identified three classes of pathogen virulence genes that are useful for interpreting our microarray data with *E. chrysanthemi*. Class I virulence genes ("true virulence genes") are directly involved with host interactions, damage the host, are not present in nonpathogenic strains, and frequently are targets for host resistance surveillance. Class I virulence factors include low- and high-molecular-weight toxins, enzymes that attack the structural integrity of host tissue, and damaging proteins that are directed at or into host cells by various secretion systems. Wassenaar and Gaastra also classify class II virulence genes that code for factors which regulate expression of true virulence genes, activate virulence factors by modification or processing, or are required in other ways for the activity of class I factors, such as specific proteases, methylases, chaperones, and so on. Finally, class III virulence genes encode proteins that compromise host defense, cytoskeletal structure, intracellular signaling, or other essential activities, and also include specific transporters, red ox protectant systems, xenobiotic degraders, osmosensors, pH regulators, and other devices required for the pathogen to cope with the spartan and generally hostile environments found within hosts. One major distinction is that, unlike true housekeeping genes, class III virulence genes are expected to be up-regulated in the host environment compared with growth of the pathogen on laboratory culture media.

Table 1. Characteristics of plant down-regulated *Erwinia chrysanthemi* 3937 clones

Clone ID[a]	P/C ratio[b]	Gene[c]	Homologous gene (organism)/protein ID[d]	Expectation	Feature of homologous gene
ECH109	0.7	1	*cyoB* (*E. coli*)/P18401	2e-93	Cytochrome o ubiquinol oxidase
		2	*cyoE* (*E. coli*)/P18404	3e-49	CyoE/heme O synthase
ECH117	0.7	1	*ubiB* (*E. coli*)/P27854	0	Unknown protein
		2	*ubiE* (*E. coli*)/P27851	5e-50	Ubiquinone or menaquinone synthesis
ECH120	0.6	1	PA0283 (*Pseudomonas aeruginosa*)/C83610	6e-77	Sulfate-binding protein precursor
(2.95 kb)		2	*cysT* (*Pseudomonas aeruginosa*)/B83610	0	Sulfate transport protein
		3	*cysW* (*Pseudomonas aeruginosa*)/A83610	0	Sulfate transport protein
		4	*cysA* (*Pseudomonas aeruginosa*)/H83609	6e-91	Sulfate transport protein
ECH212	0.5	1	*metA* (*E. coli*)/P07623	4e-46	Methionine biosynthesis
		2	*rrnD* (*Salmonella typhimurium*)/NA	0	23S rRNA
ECH235	0.6	1	YPO2876 (*Yersinia pestis*)/NP 406381.1	9e-32	Putative lipoprotein
(3.15 kb)		2	*yfgK* (*E. coli*)/P77254	0	Putative GTP-binding factor
		3	YPO2873 (*Yersinia pestis*)/NP 406378.1	4e-12	Unknown protein
		4	*prt* (*Erwinia carotovora*)/Q99132	4e-76	Extracellular protease
ECH302	0.4	1	**rpoB* (*Salmonella typhimurium*)/AAF33499.1	0	DNA-directed RNA polymerase
		2	**rpoC* (*E. coli*)/AAC43086.1	8e-87	DNA-directed RNA polymerase, β-subunit
ECH312	0.3	1	**rpoC* (*E. coli*)/AAC43085.1	0	DNA-directed RNA polymerase, β-subunit
		2	**rpoB* (*Salmonella typhimurium*)/AAF33499.1	5e-93	DNA-directed RNA polymerase
ECH317	0.3	1	**prlA* (*E. coli*)/P03844	0	Preprotein translocase, SecY subunit
		2	**rplF* (*E. coli*)/CAA25720.1	4e-15	50S ribosomal subunit protein L6
ECH327	0.4	1	*yheM* (*E. coli*)/P45531	4e-42	Unknown protein
		2	*fusA* (*Salmonella typhimurium*)/P26229	0	Elongation factor G (see ECH411)
ECH402	0.4	1	*ytaP* (*Bacillus subtilis*)/B69988	4e-4	Unknown protein
		2	*aceF* (*E. coli*)/P06959	0	Dihydrolipoamide acetyltransferase
ECH407	0.4	1	**rpsK* (*E. coli*)/NP 289858.1	4e-35	30S ribosomal subunit protein 11S
		2	**yhdN* (*E. coli*)/P36677	7e-29	Unknown protein
ECH409	0.6	1	*acnB* (*E. coli*)/P36683	0	Aconitate hydratase 2
ECH410	0.3	1	*rpoC* (*E. coli*)/AAC43086.1	5e-83	DNA-directed RNA polymerase, β-subunit
ECH411	0.5	1	*fusA* (*Salmonella typhimurium*)/P26229	0	Elongation factor G (see ECH327)
		2	*bfd* (*Yersinia pestis*)/NP 403857.1	3e-19	Bacterioferritin-associated ferredoxin
		3	*bfr* (*Serratia marcescens*) O68935	6e-43	Bacterioferritin
ECH412	0.5	1	No matching gene		

[a] Clone inserts for which molecular sizes are shown were completely sequenced.

[b] Arrays of *E. chrysanthemi* 3937 random genomic clones (approximately 3-kb inserts amplified by polymerase chain reaction) were hybridized with plant- or culture-probe. The hybridization signal intensity obtained with plant-probe was divided by that from culture-probe in order to obtain the P/C ratio.

[c] Expected gene number in a candidate clone based on BLASTX, BLASTP, and BLASTN searches (version 2.0 at the NCBI site). The BLAST searches were done against all GenBank sequences. Each gene number corresponds to one gene.

[d] Genes with the highest scores in the blast searches are listed. The asterisks in a clone indicate the same gene organization as in *Escherichia coli*. NA = not available.

Table 2. Characteristics of plant up-regulated *Erwinia chrysanthemi* 3937 clones

Clone ID[b]	P/C ratio[a] 3 kb[c]	ORF[d]	Gene[e]	Homologous gene (organism)/protein ID[f]	Expectation	Feature of homologous gene
ECH1	2.4		1	PM0935 (*Pasteurella multocida*)/AAK03019.1	1e-52	Unknown protein
(2.84 kb)		2.1	2	**+** *purU* (*E. coli*)/ P37051	0	Formyltetrahydrofolate deformylase
			3	*ychJ* (*E. coli*)/ P37052	1e-19	Unknown protein
			4	*rssA* (*Erwinia chrysanthemi*)/CAC44354.1	0	Two-component response regulator
ECH3	1.2		1	*yxeQ* (*Bacillus subtilis*)/P54956	1e-60	Unknown protein
(2.84 kb)			2	N/A (*Salmonella typhimurium*)/AAC15463.1	8e-18	Putative *XylS/AraC* transcriptional activator
		2.8	3	*pfeA* (*Pseudomonas aeruginosa*)/Q05098	6e-61	Ferric enterobactin receptor
ECH5	1.3		1	ORF4 (*Acinetobacter* sp.)/CAA05331.1	1e-20	Acyl-CoA dehydrogenase
(3.06 kb)			2	CAC2192 (*Clostridium acetobutylicum*)/NP 348809.1	2e-4	Lactoylglutathione lyase
		1.3	3	*surfAB* (*Bacillus subtilis*)/Q04747	4e-31	Surfactin synthase subunit 2
ECH6	2.2	1.8	1	*fhuA* (*E. coli*)/P06971	6e-97	Ferrichrome-iron receptor
(2.97 kb)			2	*rsmC* (*Erwinia carotovora*)/AAF01688.1	4e-10	Global regulatory protein
ECH7	1.7	2.1	1	**+** *yajO* (*E. coli*)/P77735	5e-46	NAD(P)H-dependent xylose reductase
(3.08 kb)			2	*ompA* (*E. coli*)/P02936	2e-41	Outer membrane protein A
ECH9	7.0		1	*aldB* (*E. coli*)/P37685	0	Aldehyde dehydrogenase B
(2.74 kb)		1.5	2	*pivMB* (*Moraxella bovis*)/P20665	4e-35	DNA inversion/pilin gene inverting protein
			3	*xylB* (*E. coli*)/P09099	3e-22	Xylulokinase
ECH10	1.8	3.9	1	CC2809 (*Caulobacter crescentus*)/AAK24773.1	3e-63	Peptidase
(2.84 kb)			2	N/A (*Mycobacterium tuberculosis*)/B70686	4e-32	Unknown protein
			3	*yeiW* (*E. coli*)/P58039	3e-31	Unknown protein
			4	*yeiP* (*E. coli*)/P33028	2e-92	Putative elongation factor P
ECH11	2.7	1.6	1	*pepT* (*E. coli*)/P29745	0	Putative peptidase T
(2.56 kb)		1.6	2	*feoB* (*E. coli*)/P33650	3e-42	Ferrous iron transport protein B
ECH12	5.4		1	**yfjB* (*E. coli*)/P37768	7e-27	Probable inorganic polyphosphate/ATP-NAD kinase
(2.44 kb)		2.6	2	* **+** *recN* (*E. coli*)/P05824	0	Recombination and DNA repair
			3	**smpA* (*E. coli*)/P23089	2e-25	Unknown ORF, membrane protein
ECH13	1.8		1	*mutL* (*E. coli*)/P23367	8e-31	Mismatch repair protein
		1.2	2	*hflX* (*E. coli*)/P25519	7e-80	GTP-binding protein
ECH14	2.9		1	AGR pAT 127 (*Agrobacterium tumefaciens*)/NP 396022.1	5e-9	Unknown protein
(3.29 kb)			2	ECs3633 (*E. coli*)/NP 311660.1	0	Unknown protein
			3	*ygcM* (*E. coli*)/Q46903	2e-62	Putative 6-pyruvoyl tetrahydrobiopterin synthase
		1.8	4	*picA* (*Agrobacterium tumefaciens*)/P27644	2e-13	Polygalacturonase
ECH15	9.5		1	*yhdY* (*E. coli*)/P45768	5e-23	Hypothetical amino acid ABC transporter
(2.51 kb)			2	*gltJ* (*E. coli*)/P41074	1e-21	Glutamate/aspartate permease
		2.6	3	**+** *ydfG* (*E. coli*)/P39831	5e-32	Putative oxidoreductase
			4	No matching gene		
ECH16 (1.20 kb)	13.6		1	*pps2* (*Bacillus subtilis*)/P39846	1e-90	Peptide synthase 2
ECH110	1.8	2.2	1	*ggt* (*E. coli*)/P18956	2e-18	Gamma-glutamyltranspeptidase
(2.75 kb)			2	*tsr* (*E. coli*)/P02942	1e-62	Methyl-accepting chemotaxis protein I
			3	*mccF* (*Vibrio cholerae*)/NP 232833.1	4e-11	Microcin self-immunity protein
ECH111	1.4	W	1	CC0481 (*Caulobacter crescentus*)/P58112	7e-22	Pirin-related protein (Apoptosis)
(3.22 kb)		2.4	2	**+** *nox* (*Enterococcus faecalis*)/P37061	4e-14	NADH oxidase (reduces $O_2 \rightarrow H_2O$)
			3	*ycfD* (*E. coli*)/P27431	9e-65	Unknown protein
ECH123	1.2		1	*dgoT* (*E. coli*)/NP 418146.1	4e-26	D-Galactonate transport
(2.68 kb)		5.2	2	*perR* (*E. coli*)/Q57083	7e-36	Peroxide resistance protein (LysR regulator)
		4.0	3	*yfeA* (*Yersinia pestis*)/Q56952	3e-61	Periplasmic chelated iron-binding protein
ECH124	1.6		1	*talB* (*Salmonella typhimurium*)/AAL18971.1	2e-6	Transaldolase B
			2	*ycdT* (*E. coli*)/P75908	7e-26	Unknown protein
			3	*mogA* (*E. coli*)/1DI6A	2e-90	Molybdenum cofactor biosynthesis protein, MogA
			4	YPO0465 (*Yersinia pestis*)/NP 404107.1	4e-5	Putative transporter
ECH125	1.8		1	YPO1773 (*Yersinia pestis*)/NP 405340.1	0	Para-aminobenzoate synthase component
			2	STY1956 (*Salmonella enterica*) / CAD05509.1	0	L-serine deaminase 1

(*continued on next page*)

[a] Hybridization signal intensity obtained with plant-probe was divided by that from culture-probe in order to obtain the P/C ratio

[b] Clone inserts for which molecular sizes are shown were completely sequenced.

[c] Arrays of *E. chrysanthemi* 3937 random genomic clones (approximately 3-kb inserts amplified by polymerase chain reaction) were hybridized with plant- or culture-probe.

[d] Individual open reading frames (ORFs) identified in the plant up-regulated clones were polymerase chain reaction amplified (0.4 to 1.6 kb partial ORF fragments), re-arrayed, and hybridized with plant-probe and culture-probe. W = signals too weak to obtain reliable P/C ratios.

[e] Expected gene number in a candidate clone based on BLASTX, BLASTP, and BLASTN searches (version 2.0 at the NCBI site). The BLAST searches were done against all GenBank sequences. Each gene number corresponds to one gene.

[f] Genes with the highest scores in the blast searches are listed. The asterisks in a clone indicate the same gene organization as in *Escherichia coli*; + = genes which were mutated by Tn5 insertion for virulence assays. NA = not available.

Table 2. (*continued from preceding page*)

Clone ID[b]	P/C ratio[a] 3 kb[c]	ORF[d]	Gene[e]	Homologous gene (organism)/protein ID[f]	Expectation	Feature of homologous gene
ECH126	2.3		1	*yfeC* (*Yersinia pestis*)/Q56954	3e-67	Chelated iron transport
(2.91 kb)		2.4	2	*yfeD* (*Yersinia pestis*)/Q56955	7e-90	Chelated iron transport
			3	*mauR* (*Klebsiella pneumoniae*)/P52684	0	Malonate utilization transacting regulator
			4	*fabD* (*Haemophilus influenzae*)/P43712	5e-5	Malonyl-CoA-acyl carrier protein transacylase
ECH128	2.4		1	orfA (*Klebsiella pneumoniae*)/AAK29065.1	2e-5	IS element (see ECH224)
			2	PA1408 (*Pseudomonas aeruginosa*)/G83469	5e-27	Unknown protein
ECH129	2.5		1	*ygaA* (*E. coli*)/P37013	4e-41	Putative transcriptional regulator
(2.73 kb)		5.3	2	+ *roo* (*Desulfovibrio gigas*)/Q9F0J6	2e-78	Rubredoxin oxidase-rubredoxin fusion
			3	*rubB* (*Acinetobacter calcoaceticus*)/P42454	0	Rubredoxin-NAD(+) reductase
ECH130	5.8		1	*ybgJ* (*E. coli*)/P75744	5e-7	Unknown protein
(2.41 kb)		2.6	2	RSp0931 (*Ralstonia solanacearum*)/NP 522492.1	5e-78	Amino acid binding periplasmic protein, transporter
			3	SMc02371 (*Sinorhizobium meliloti*)/NP 385133.1	1e-48	Unknown protein
ECH131	5.2		1	*ybjT* (*E. coli*)/P75822	1e-23	Putative dTDP-glucose enzyme
(3.27 kb)		9.8	2	*hcr* (*Yersinia pestis*)/CAC90188.1	0	Putative oxidoreductase (Ferredoxin)
		10.4	3	+ *hcp* (*Yersinia pestis*)/CAC90189.1	0	Prismane homolog (only exp. during anaerobic growth)
ECH136	1.3		1	PA3782 (*Pseudomonas aeruginosa*)/F83172	6e-22	Probable transcriptional regulator
			2	*hipA* (*E. coli*)/P23874	1e-50	DNA biosynthesis regulation
ECH139	1.6	1.0	1	*y4op* (*Sinorhizobium fredii*)/AAC02677.1	2e-44	Suger ABC transporter binding protein
(3.11 kb)		2.0	2	*yceE* (*E. coli*)/P25744	0	Drug MFS-transporter, fosfomycin&deoxycholate
ECH140	2.2		1	lin2949 (*Listeria innocua*)/CAC98174.1	7e-21	Putative transport protein
(1.48 kb)			2	*ytfD* (*Bacillus subtilis*)/F69991	5e-55	N-Acylamino racemase homolog
ECH142	1.9	1.8	1	*xenB* (*Pseudomonas aeruginosa*)/D83102	4e-87	Xenobiotic reductase; Fe protein
(3.03 kb)			2	*TnpA1* (*Pseudomonas fluorescens*)/CAB87819.1	3e-78	Transposase
			3	*ydiI* (*E. coli*)/P77781	3e-48	Unknown protein
		1.9	4	*entA* (*E. coli*)/P15047	8e-49	Enterobactin synthesis
ECH146	1.9	4.8	1	+ *ydgD* (*E. coli*)/P76176	3e-79	Protease
(2.56 kb)		42.3	2	+ No matching gene		Unknown lysine-rich protein
			3	*rstB* (*E. coli*)/P18392	3e-36	Histidine sensor kinase
ECH147	1.6		1	No matching gene		
			2	*ycdW* (*E. coli*)/P75913	2e-10	Putative 2-hydroxyacid dehydrogenase
ECH202	2.2	11.4	1	cds106 (*E. coli*)/CAA50734.1	3e-35	Putative peptidase
			2	*yddB* (*E. coli*)/P31827	1e-18	Unknown protein
ECH205	5.4		1	*trbI* (*Ralstonia eutropha*)/CAA71794.1	1e-32	Conjugative transfer protein
(2.53 kb)			2	+ *pps1* (*Bacillus subtilis*)/P39845	9e-49	Peptide synthase 1
ECH206	5.1	W	1	NA (*Mus musculus*)/P49025	0.003	CITRON protein
ECH207	2.7		1	*sucA* (*E. coli*)/NP 286442.1	0	2-Oxoglutarate dehydrogenase
ECH209	6.2	1.8	1	*xenB* (*Pseudomonas aeruginosa*)/D83102	4e-87	Xenobiotic reductase; Fe protein
(2.66 kb)			2	*TnpA1* (*Pseudomonas fluorescens*)/CAB87819.1	3e-78	Transposase
			3	*ydiI* (*E. coli*)/P77781	3e-48	Unknown protein
ECH210	2.2		1	*oppD* (*E. coli*)/P76027	2e-99	Oligopeptide transporter, ATP-binding protein
(3.18 kb)			2	*oppF* (*E. coli*)/P77737	0	Oligopeptide transporter, ATP-binding protein
			3	*yciU* (*E. coli*)/P76028	6e-55	Unknown protein
		1.7	4	*cls* (*E. coli*)/P31071	0	Cardiolipin synthase (see ECH229 and 234)
ECH211	4.5		1	+ *syrE* (*Pseudomonas syringae*)/T14593	2e-57	Peptide synthase
ECH215	1.6		1	YPO0874 (*Yersinia pestis*)/NP 404494.1	0	Unknown protein
ECH217	2.3	2.2	1	+ *ptsI* (*Salmonella typhimurium*)/P12654	0	Phosphoenolpyruvate-protein phosphotransferase (PTS)
(2.88 kb)			2	*crr* (*E. coli*)/P08837	3e-78	Glucose-specific IIA component (PTS)
		2.9	3	PA2713 (*Pseudomonas aeruginosa*)/A83307	1e-13	Unknown protein
ECH219	3.6		1	*iluK* (*Klebsiella pneumoniae*)/P27696	0	Acetolactate synthase
			2	*aldC* (*Enterobacter aerogenes*)/P05361	4e-70	alpha-acetolactate decarboxylase
		W	3	*budR* (*Klebsiella terrigena*)/P52666	7e-49	Bud operon transcriptional regulator
ECH220	2.3	4.4	1	*yxjG* (*Bacillus subtilis*)/P42318	3e-83	Unknown protein
(3.45 kb)		1.1	2	*brnQ* (*E. coli*)/P37011	0	branched-chain amino acid transporter
			3	NA (*Salmonella typhimurium*)/S35983	6e-96	Proline permease
ECH222	4.2		1	*tar* (*E. coli*)/P07017	3e-20	Methyl-accepting chemotaxis protein II
(2.61 kb)			2	No matching gene		
		1.2	3	orf8 (*Pseudomonas denitrificans*)/P29941	9e-27	Hypothetical cob gene
ECH223	1.8	2.4	1	*estV* (*E. chrysanthemi* 3937)/NA	0	Esterase
(2.79 kb)			2	STY2675 (*Salmonella enterica*)/CAD07669.1	1e-72	Putative amidotransferase
			3	PA0163 (*Pseudomonas aeruginosa*)/A83625	1e-23	Putative transcriptional regulator
ECH224	2.6		1	*valS* (*E. coli*)/P07118	0	Valyl-tRNA synthetase
			2	orfA (*Klebsiella pneumoniae*)/AAK29065.1	2e-50	Unknown protein (see ECH128)
			3	orfB (*Klebsiella pneumoniae*)/AAK29066.1	6e-19	Unknown protein (see ECH128)

Our microarray results with *E. chrysanthemi* suggest that class III virulence genes far outnumber class I and II genes and raise the specter that they are more important than previously thought for growth of the pathogen in a plant host. Although a few class III virulence genes have previously been identified in *E. chrysanthemi* (Hassouni et al. 1999; López-Solanilla et al. 2001; Nachin et al. 2001), our results suggest that many more are present and may contribute to virulence.

We successfully used an in vitro Tn*5* insertion approach for rapidly constructing mutations in genes on the microarray clones and then recombining them into the *E. chrysanthemi* chromosome. Although results are as yet limited, mutations in 3 of 17 plant up-regulated genes were reduced in virulence on African violet leaves (Fig. 2). These include the *purU* homo-

logue in ECH1 and the *pheC* homologue in ECH233, both appearing to be class III virulence genes. While we do not understand the function of these genes in promoting virulence, the homologues have known biochemical functions. In addition, mutation of a class I peptide synthase homologue in ECH205 led to reduced virulence, perhaps indicating that a previously unknown cyclic peptide contributes to virulence. Significantly, mutation of the peptide synthase homologue in clone ECH211 did not appear to affect virulence, suggesting that *E. chrysanthemi* may contain at least two different peptide synthase genes. Based on bacterial growth on M9 glucose minimal medium, only the *purU* mutant out of three reduced virulence strains was revealed to be auxotrophic (unpublished data). This auxotrophic phenotype may have contributed to the re-

Table 2. (*continued from preceding page*)

Clone ID[b]	P/C ratio[a]		Gene[e]	Homologous gene (organism)/protein ID[f]	Expectation	Feature of homologous gene
	3 kb[c]	ORF[d]				
ECH225	2.5	6.7	1	+ PA5395 (*Pseudomonas aeruginosa*)/C82971	1e-61	Unknown protein
(2.37 kb)		2.4	2	+ *estV* (*E. chrysanthemi* 3937)/NA	0	Esterase
			3	*yfeJ* (*Salmonella typhimurium*)/P40194	2e-9	Unknown protein
ECH226	2.9		1	*ygbD* (*E. coli*)/P37596	5e-53	Rubredoxin reductase
(2.72 kb)		4.1	2	*tehB* (*E. coli*)/P25397	7e-62	Tellurite resistance
			3	*ribB* (*E. coli*)/P24199	1e-92	Riboflavin, FMN and FAD synthesis
ECH228	4.8		1	*ydhM* (*E. coli*)/P76425	1e-38	Unknown His rich protein
(2.90 kb)			2	*argG* (*E. chrysanthemi*)/AAF74776.1	0	Argininosuccinate synthase
			3	*pecS* (*E. chrysanthemi*)/P42195	0	Regulator of pectinase, cellulase, etc.
ECH229	3.1	W	1	*ybhO* (*E. coli*)/P75771	2e-48	Cardiolipin synthase (see ECH210)
(2.67 kb)			2	*pivMB* (*Moraxella bovis*)/P20665	5e-34	DNA inversion/pilin gene inverting protein
			3	*ybhL* (*E. coli*)/P75768	5e-95	Unknown protein
ECH230	2.9	1.8	1	*xenB* (*Pseudomonas aeruginosa*)/D83102	9e-36	Xenobiotic reductase; Fe protein
			2	*TnpA1* (*Pseudomonas fluorescens*)/CAB87819.1	3e-78	Transposase
			3	*ydiI* (*E. coli*) / P77781	1e-34	Unknown protein
ECH231	1.4		1	Z3973 (*E. coli* O157:H7)/BAB36957.1	9e-33	Putative transcriptional regulator
ECH233 (0.61 kb)	3.3		1	+ *pheC* (*Pseudomonas aeruginosa*)/Q01269	8e-64	Cyclohexadienyl dehydratase (phenylalanine synthesis)
ECH234	1.5		1	*ybhO* (*E. coli*)/P75771	2e-29	Cardiolipin synthase (see ECH210 and 229)
			2	*ybhL* (*E. coli*)/P75768	1e-32	Unknown protein
ECH237	1.5		1	**recF* (*E. coli*)/P03016	3e-73	ssDNA and ds DNA binding, ATP binding
(2.98 kb)			2	**gyrB* (*E. coli*)/P06982	0	DNA gyrase subunit B, typeII topoisomerase
ECH238	1.4		1	No matching gene		
(3.20 kb)			2	SCE20.35. (*Streptomyces coelicolor*)/CAB65591.1	1e-6	Unknown protein
			3	*yddH* (*E. coli*)/P76121	2e-32	Unknown protein
			4	*ssb* (*E. coli*)/P02339	3e-52	ssDNA binding
ECH239	3.2	9.1	1	NA (*Saccharomyces cerevisiae*)/P53750	4e-42	Unknown protein
(2.78 kb)		2.6	2	+ SCE59.14c (*Streptomyces coelicolor*)/CAB72201.1	1e-11	Unknown protein
			3	PA3034 (*Pseudomonas aeruginosa*)/A83267	5e-27	Putative transcriptional regulator
		6.7	4	PA5395 (*Pseudomonas aeruginosa*)/C82971	1e-61	Unknown protein
ECH240	4.8		1	*wcaJ* (*E. coli*)/P71241	8e-80	Putative colanic acid synthesis
(2.66 kb)			2	VC0935 (*Vibrio cholerae*)/E82262	1e-18	Unknown protein
			3	*wza* (*E. coli*)/P76388	2e-14	Polysaccharide export protein
ECH241	2.6		1	*oxlT* (*Halobacterium* sp.)/AAG18775.1	3e-10	Oxalate/formate antiporter
			2	*oxc* (*E. coli*)/P78093	8e-7	Probable oxalyl-CoA decarboxylase; oxalate catabolism
			3	b2374 (*E. coli*)/P77407	6e-44	Unknown protein
ECH242	2.7	2.3	1	*togB* (*E. chrysanthemi*)/CAC44121.1	0	Transport of oligogalacturonides and chemotaxis
			2	*kdgM* (*E. chrysanthemi*)/CAC86224.1	0	Oligogalacturonate specific porin
		2.1	3	*paeX* (*E. chrysanthemi*)/NA	0	Pectin acetylesterase
ECH243	3.9		1	*trbI* (*Ralstonia eutropha*)/CAA71794.1	7e-28	Conjugative transfer protein
			2	*relB* (*Vibrio cholerae*)/NP 232745.1	6e-8	Unknown protein
			3	No matching gene		
			4	NA (*E. coli*)/AAF76755.1	6e-6	Putative transposase, IS30
ECH244	3.2		1	No matching gene		
			2	*int* (*E. coli*)/AAF06962.1	2e-72	P4 integrase-like protein
			3	*yjgQ* (*E. coli*)/P39341	5e-44	Unknown protein
ECH245	2.5		1	*syd* (*Yersinia pestis*)/NP 404648.1	2e-60	Syd protein (interact with SecY)
			2	YPO1033 (*Yersinia pestis*)/NP 404646.1	2e-22	Conserved hypothetical protein
			3	*ygdH* (*E. coli*)/NP 289350.1	0	Unknown protein

(*continued on next page*)

duced virulence. Nevertheless, it is possible that the *purU* mutation conferred true reduced virulence because mutation of the *Salmonella typhimurium ptsI* homologue (Table 2), which also was auxotrophic, retained full virulence. Mutation of the *P. aeruginosa pheC* homologue (ECH233) in *E. chrysanthemi* resulted in a prototrophic phenotype on M9 medium but was reduced in virulence. In *P. aeruginosa,* this gene encodes periplasmic prephenate dehydratase (cyclohexadienyl dehydratase), a phenylalanine biosynthetic pathway member (Calhoun et al. 2001). The physiological role for this enzyme in *E. chrysanthemi* is not understood, but the *pheC* homologue would appear to represent a class III virulence gene.

Class III virulence genes generally have been neglected to date, perhaps because investigators have tended to focus on class I virulence genes that directly damage hosts. Undoubtedly, many class III genes are redundant or overlapping in function, so they might not be revealed as virulence-impaired in traditional mutant screens. Indeed, in vivo expression approaches such as microarrays probably are required to reveal the presence of many class III virulence genes. Although our results are novel for a bacterial plant pathogen, investigators with plant (Boch et al. in press) and animal (Smith 2000) pathogens have emphasized the use of in vivo expression approaches for the study of class III virulence genes. As the genome sequence of *E. chrysanthemi* is completed, more extensive microarray gene expression data is accumulated, and relevant mutant strains are constructed, our understanding of class III virulence genes will expand.

Several of the plant up-regulated genes observed in the array experiments are associated with anaerobic growth. For instance, a ferrous iron transporter homologue of the *Escherichia coli feoB* gene was plant up-regulated in *Erwinia*

Table 2. (*continued from preceding page*)

Clone ID[b]	P/C ratio[a]		Gene[e]	Homologous gene (organism)/protein ID[f]	Expectation	Feature of homologous gene
	3 kb[c]	ORF[d]				
ECH246	2.7		1	NA (*Haemophilus influenzae*)/NP 043506.1	1e-47	Phage HP1; orf35
			2	NA (*Haemophilus influenzae*)/AAK37818.1	3e-24	Phage HP2; orf34
			3	*tfa* (*E. coli*)/NP 037719.1	1e-31	Gp29 of bacteriophage HK97
			4	N/A (*E. coli*)/S18689	6e-24	Phage P1 major tail fiber protein S
ECH307	5.5		1	J (Enterobacteria phage P2)/NP 052269.1	8e-67	Major tail sheath protein (FI)
(2.71 kb)			2	STY1639 (*Salmonella enterica*)/CAD01885.1	1e-30	Tail fiber assembly protein homolog from prophage E14
			3	NA (*E. coli*)/S18688	1e-39	Sc/SvO, phage P1 major tail fiber protein S
			4	NA (*Erwinia carotovora*)/BAA76528.2	2e-53	DNA-invertase (also *E. coli* BAB38415.1) (see ECH502)
ECH308	3.9	42.3	1	No matching gene		Unknown lysine-rich protein
			2	*rstB* (*E. coli*)/P18392	3e-36	Histidine sensor kinase
			3	*rstA* (*E. coli*)/P52108	9e-72	Transcriptional regulator
ECH309	2.2		1	*atsC* (*Pseudomonas putida*)/AAD31787.1	6e-40	ATP binding; sulfate ester transporter
(3.38 kb)		3.5	2	PA2336 (*Pseudomonas aeruginosa*)/NP 251026.1	9e-81	Unknown protein
ECH314	3.5	4.8	1	*ydgD* (*E. coli*)/P76176	3e-79	Protease
		42.3	2	No matching gene		Unknown lysine-rich protein
			3	*rstB* (*E. coli*)/P18392	3e-36	Histidine sensor kinase
ECH316	4.4		1	CC1790 (*Caulobacter crescentus*)/AAK23766.1	2e-14	Unknown protein
(3.36 kb)		2.4	2	*ahpF* (*E. coli*)/P35340	0	Alkyl hydroperoxide reductase-Peroxide degrader
			3	*ahpC* (*Salmonella typhimurium*)/P19479	3e-95	Alkyl hydroperoxide reductase C22
		1.0	4	*hmuU* (*Yersinia pestis*)/Q56992	2e-24	ABC transporter (hemin ABC transporter in *Dienococcus*)
ECH323	5.1		1	*ydbH* (*E. coli*)/P52645	0	Unknown protein
(2.62 kb)			2	*ynbE* (*E. coli*)/P76075	4e-12	Unknown protein
		2.9	3	+ *acpD* (*E. coli*)/P41407	3e-84	Acyl carrier protein phosphodiesterase
ECH404	4.7	1.7	1	*cysZ* (*E. coli*)/P12610	1e-85	Sulfate transporter
(1.51 kb)		16.7	2	*cysK* (*Salmonella typhimurium*)/P12674	2e-85	Cysteine synthase A
ECH405	5.8	4.8	1	*ydgD* (*E. coli*)/P76176	3e-79	Protease
		42.3	2	No matching gene		Unknown lysine-rich protein
			3	*rstB* (*E. coli*)/P18392	3e-36	Histidine sensor kinase
ECH406	2.2	1.9	1	*speD* (*Yersinia pestis*)/CAC92642.1	2e-92	S-adenosylmethionine decarboxylase
(2.87 kb)			2	*nrdI* (*Salmonella typhimurium*)/Q56109	2e-38	Putative ribonucleotide reductase
		0.8	3	*nrdE* (*E. coli*)/P39452	0	Ribonucleoside-diphoshpate reductase 2, alpha subunit
ECH408	2.1		1	*pelE* (*E. chrysanthemi*)/P18210	0	pectate lyase
			2	*pelD* (*E. chrysanthemi*)/P18209	0	pectate lyase
ECH502	7.6		1	STY1639 (*Salmonella enterica*)/CAD01885.1	1e-30	Tail fiber assembly protein homolog
(3.62 kb)			2	J (Enterobacteria phage P2)/NP 052269.1	0	Major tail sheath protein FI (see ECH307)
			3	I (Enterobacteria phage P2)/NP 052270.1	3e-77	P2 tail tube protein FII
			4	E (Enterobacteria phage P2)/NP 046781.1	5e-21	Essential tail protein gpE
			5	No matching gene		
ECH503	2.7	1.6	1	*ptsG* (*E. coli*)/P05053	0	PTS system, glucose-specific IIBC component (see ECH217)
(2.61 kb)		1.9	2	N/A (*Ralstonia eutropa*)/Q07251	9e-45	L-lactate dehydrogenase
ECH506	1.7		1	*ibpA* (*E. coli*)/P29209	7e-48	Heat shock protein A
(2.66 kb)		3.5	2	*ibpB* (*E. coli*)/P29210	3e-49	Heat shock protein B
		1.0	3	SODiT1 (*Spinacia oleracea*)/Q41364	1e-78	2-Oxoglutarate/malate translocator
Controls						
pelD ORF	19.3			(*E. chrysanthemi*)/CAA10570.1	0	Pectate lyase
hrpG ORF	5.3			(*E. chrysanthemi*)/AAC31974.1	0	Component of Hrp secretion system
hrpN ORF	7.2			(*E. chrysanthemi*)/CAC20124.1	0	Harpin elicitor

chrysanthemi (ECH11). Although we do not know if this gene is functional, it is of interest that, in *Escherichia coli,* the *feoAB* genes contribute to iron supply under anaerobic conditions and *feo* mutants are impaired in virulence on mouse (Kammler et al. 1993). The prismane protein homologue (ECH131) is only expressed in *E. coli* and other facultative anaerobes under anaerobic conditions, and has been suggested to have a role in nitrate/nitrite respiration (van den Berg et al. 2000). A ferredoxin gene was linked to the prismane gene in ECH131. This protein is believed to be involved in assembly of Fe-S reduction proteins in bacteria (Nakamura et al. 1999). Further, a rubredoxin oxidase/rubredoxin hybrid protein gene and an adjacent, probably cotranscribed rubredoxin reductase gene, were plant up-regulated in *Erwinia chrysanthemi* (ECH129). This raises the possibility that several Fe-S redox proteins may establish an electron shuttle from NADH to oxygen, generating water and reducing the ambient oxygen concentration (Gomes et al. 1997). A NOXase homologue on ECH111 also uses NADH to reduce oxygen directly to water. Finally, it is interesting that several of the plant down-regulated genes in *E. chrysanthemi* are involved in oxidative phosphorylation, perhaps an indication of conversion to microaerobic growth in the host. Soft-rotting *Erwinia* spp. have been known to undergo microaerobic conditions in fleshy host tissues such as potato tuber tissues (Perombelon and Kelman 1980; Vayda et al. 1992). It has not been realized that anaerobic conditions also may form in normally infected leaf tissues, but this possibility should be investigated further.

Despite our interesting results in microarray experiments with PCR-amplified clone inserts, several limitations were observed. The complete genome sequence of *E. chrysanthemi* was not available; therefore, random clone inserts were PCR amplified and arrayed. This approach would fail to reveal any uncloneable genes and some genes may have been over-represented in the library. Clones such as ECH146, ECH308, ECH314, and ECH405 and ECH142, ECH209, and ECH230 encompassed essentially the same DNA segments, whereas most plant up-regulated clones recovered were unique. Therefore, it is likely that we missed several genes in the screen. Recent shotgun sequencing data obtained in the *E. chrysanthemi* genome project have estimated the genome size as approximately 5.5 Mb, which is much larger than expected. Therefore, our 5,000 array clones gave approximately 2.7× coverage of the genome. We also may have missed some genes not expressed at the 14-h sampling time or for which mRNA turnover was rapid during the infection process, because we selected clones according to reproducibility of the P/C expression ratio in replicated experiments using several batches of bacterial RNA. Most plant up-regulated clones also contained more than one ORF. Thus, microarray expression signals were averages of the ORFs on a clone, with a worst scenario being compensating plant up- and down-regulated genes on a single clone leading to little or no net change in gene expression. Such a clone naturally would not be selected for further study. Further, when single ORFs were PCR amplified and re-arrayed, it was often disclosed that their P/C expression ratios were greater than the parent clone. In most cases, this resulted from the presence of a relatively highly expressed constitutive gene on a clone in addition to the plant up-regulated gene. The consequent dilution effect led to a decrease in the observed P/C induction ratio for the entire clone insert (Table 2). The use of clones in the project also necessitated considerable DNA sequencing. Individual ORFs then had to be PCR amplified and re-arrayed to positively identify plant up-regulated genes, involving considerable time and expense. We also experienced some problems with amounts of PCR clone inserts arrayed. Variation occurred with hybridization signals between independent arrays and necessitated careful replication of results. Considerable work also was required to recover sufficient bacteria from plant leaves, extract RNA, and prepare labeled cDNAs.

Due to the effort required to analyze clones in the array experiments, we only extracted RNA from bacteria grown in African violet leaves for 14 h. Unique temporal expression patterns can be expected for many virulence-associated genes; therefore, this may have contributed to our failure to isolate some expected genes. For instance, no plant up-regulated *hrp* genes were isolated, perhaps related to the observed early induction of these genes in *E. chrysanthemi* (M. Boccara, *personal communication*).

Most problems associated with the use of clone insert arrays in the current project will be solved by the *E. chrysanthemi* 3937 genome sequencing project, now underway. When these results are available, it will be possible to construct second-generation arrays with gene-specific sequences representing all ORFs in the genome. We also are experimenting with meth-
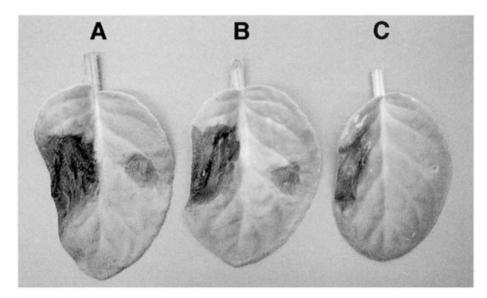

Fig. 2. Symptom phenotypes of three *Erwinia chrysanthemi* 3937 mutants with reduced virulence. Wild-type *E. chrysanthemi* 3937 or its mutant strains were inoculated into African violet (var. Katja) leaves (wild type, left; mutant, right). The photos represent symptoms at 40 h after inoculation. **A,** *Bacillus subtilis* peptide synthase homologue (ECH205) mutant; **B,** *Escherichia coli purU* homologue (ECH1) mutant; and **C,** *Pseudomonas aeruginosa pheC* homologue (ECH233) mutant.

ods permitting bacterial cDNAs to be PCR amplified before hybridizing to the arrays. PCR amplification is generally frowned upon because of the possibility of unequal amplification of cDNAs and the consequent compromise of expression data (Danchin and Sekowska 2000). Although we were able in this study to produce sufficient RNA from *E. chrysanthemi* cells grown in African violet leaves for preparation of labeled cDNA, considerable time and effort were involved. To utilize microarray global gene expression approaches with *E. chrysanthemi* in niches where bacterial numbers are very low, such as the surface of plant leaves, will require amplification of cDNAs, and we are working to achieve this.

MATERIALS AND METHODS

Plant inoculation and cell harvest.

E. chrysanthemi 3937 cells were cultured in LB medium at 28°C and harvested at an A600 of approximately 0.4. The cells were washed twice with 50 mM potassium phosphate buffer, pH 7.4, resuspended in the same buffer at 2×10^7 cells/ml, and used for plant inoculation. African violet (*Saintpaulia ionantha* var. Katja) plants were grown in soil at 28°C with a 16 h photoperiod of mixed fluorescent and incandescent lighting. The plant leaves were detached, submerged in the cell suspension, and placed in a desiccator and subjected to vacuum. After 10 min, air was readmitted into the desiccator in order to infiltrate bacteria into the leaves. Leaves were washed with distilled water and incubated at 28°C for 14 h after placing the stems in water. Then, the leaves were cut in half, placed into centrifuge tubes specifically designed for this purpose, and centrifuged at $500 \times g$ for 10 min at 2°C. Bacteria were forced from the leaves along with intercellular fluids. The resultant bacterial cell suspension was immediately centrifuged at $2,500 \times g$ for 10 min at 2°C to pellet the bacterial cells, and total RNA was extracted. In the infiltrated leaves, the bacteria multiplied approximately 180 times during the 14 h incubation.

Synthesis of fluorescent labeled cDNA from *E. chrysanthemi* 3937 total RNA.

Total *E. chrysanthemi* 3937 RNA was extracted from cells grown in culture or plant leaves immediately after pelleting with the RNAeasy Kit (Qiagen Inc., Valencia, CA, U.S.A.).

Total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at –80°C before use. For probe labeling, 6 μg of total RNA was mixed with 100 ng of random hexamer primers, heated at 70°C for 10 min, and quickly chilled on ice. The RNA sample (7 μl) was mixed with 4 μl of 5× first strand buffer (Life Technologies, Inc., Gaitherburg, MD, U.S.A.), 2 μl of 100 mM DTT, 1 μl of dNTP mix (dATP, dGTP, dCTP, each at 7.5 mM; dTTP at 3 mM), 2 μl of Cy3-dUTP (Amersham Pharmacia Biotech, Arlington Heights, IL, U.S.A.) at 25 μM, and 50 U of SuperscriptII reverse transcriptase (Life Technologies, Inc.). cDNA synthesis was performed at 45°C for 80 min in the dark and then the RNA template was hydrolyzed by incubation with 7.5 μl of 1N NaOH at 70°C for 10 min. The reaction was neutralized by adding HCl and Cy3-labeled cDNA was purified by the PCR MinElute Kit (Qiagen Inc.). Labeled probes from culture-grown cells were called culture-probe and those from plant-grown cells, plant-probe.

The vacuum infiltration method for introduction of *E. chrysanthemi* cells into African violet leaves and centrifugal recovery led to substantial amounts of high-quality total RNA from plant-grown bacteria. One hundred infiltrated leaves yielded 20 to 30 μg of bacterial RNA. More importantly, the isolated RNA did not contain detectable plant RNA, as determined by agarose gel electrophoresis (data not shown).

Preparation of *E. chrysanthemi* 3937 genomic arrays.

The *E. chrysanthemi* genome has not yet been completely sequenced; therefore, we were forced to array random genomic DNA clone inserts. *E. chrysanthemi* 3937 genomic DNA was isolated and partially digested with *Sau*3AI. The digested DNA was separated by 1% agarose gel electrophoresis and fragments (approximately 3 kb) were recovered from the gel. The genomic fragments were ligated with the plasmid vector, pBBR1MCS-4 (Kovach et al. 1995) linearized with *Bam*HI, and transformed into *Escherichia coli* DH5α cells to obtain a genomic library. Approximately 5,000 random library clones (approximately 2.7× coverage of the genome according to updated data from the *E. chrysanthemi* genome project) were independently amplified by PCR. For each clone, 25 μl of PCR reaction was used, which contained 0.4 μM M13 forward and reverse primers (5′-CCCAGTCACGACGTTGTAAAACG and 5′-AGCGGATAA-CAATTTCACAGG, respectively), 12.5 μl of 2× PreMix Buffer

Table 3. Summary of genes identified in plant down- and up-regulated clones

Gene regulation	Class	Subclass	Number[a]
Genes in plant down-regulated clones	Protein synthesis		5
	Transcription		5
	TCA cycle/oxidative phosphorylation		4
Genes in plant up-regulated clones	Transport and uptake	Iron transport or uptake	8
		Sulfate transport	2
		Amino acid or peptide transport	7
		Sugar or polysaccharide transport	6
		Other transporters	6
	Chemotaxis		3
	Anaerobiosis		6
	Regulatory proteinTranscriptional regulators		11
		Translational regulator	1
		Other regulatory protein	3
	Peptide synthesis		4
	Degrading enzyme	Pectic enzyme	4
		Proteases	6
	Defense against stress	Xenobiotic resistance	5
		Other stress genes	6
	DNA repair and recombination		6
	Transposable element		4
	Phage or phage-like component		14
	Homologous with unknown protein		44
	Open reading frame without significant homologues		10

[a] Numbers represent total gene numbers shown in Tables 1 and 2.

E (Epicentre Technologies, Madison, WI, U.S.A.) and 2.5 U Taq polymerase (Promega, Madison, WI, U.S.A.). Thirty-five cycles of denaturation at 94°C for 1 min, annealing at 55°C for 40 s, and extension at 72°C for 4 min were performed, followed by 5 min of final extension at 72°C, were carried out. The PCR products then were purified using a 96-well PCR Purification Kit (Qiagen Inc.) and finally dissolved in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The obtained DNA samples (50 to 300 ng/µl in 3× SSC) were spotted onto CMT-GAPS2 slides (Corning Inc., Corning, NY, U.S.A.) in duplicate using the GMS-417 Microarrayer (Affymetrix Inc., Santa Clara, CA, U.S.A.). In the re-array hybridization using individual ORFs (0.4 to 1.6 kb) (Table 2, column 3), all DNA samples were adjusted at 500 ng/µl in 3× SSC and spotted onto slides because small DNA fragments generally required high DNA concentration to give good hybridization signals in our hybridization condition.

Microarray hybridizations.

Labeled probes were dissolved in 40 µl of hybridization buffer (3× SSC, 25 mM HEPES, pH 7.0, 0.225% sodium dodecyl sulfate [SDS], 0.5 µg of herring testis DNA) and boiled for 2 min. The probe was immediately applied onto the genomic array slide, covered with a coverslip (Grace Bio-Labs Inc., Bend, OR, U.S.A.), and hybridized at 65°C for 16 h (at 60°C for the ORF array experiments). After hybridization, the slide was washed sequentially for 5 min each in 1× SSC–0.1% SDS, 0.1× SSC–0.1% SDS, and 0.1× SSC. The slide was dried with centrifugation and fluorescent signals were scanned by the GMS-418 Array Scanner (Affymetrix Inc). Signal strength was quantified by the ImaGene computer program (ver. 4.1; BioDiscovery, Los Angeles), setting the Empty Spot Threshold value at 2.0 to flag spots with low signal/background ratios. Background signals were subtracted using the Local-background command in the GeneSight-Lite program (BioDiscovery) and the data obtained from culture-probe or plant-probe arrays were normalized according to the total signal strength. Normalized signal intensity with plant-probe was divided by that with culture-probe to calculate the P/C ratio. P/C ratios obtained from individual hybridization experiments were averaged to give the final P/C number. Based on standard deviation calculations, clones having ≥1.5 or ≤0.66 final P/C ratios were selected as statistically significant plant up-regulated or plant down-regulated, respectively. In initial experiments, some clones with P/C ratios of approximately 1.2 to 1.4 or 0.67 to 0.8 were examined further. Hybridization experiments were repeated at least four times for each clone.

Gene knockouts in *E. chrysanthemi* 3937 and plant virulence assays.

Selected genes on plant up-regulated microarray clones were mutated by an in vitro Tn5 strategy (EZ::TN <KAN-2> Insertion Kit, Epicentre Technologies). Insertions were physically mapped and precisely located by sequencing from transposon primers. The plasmid constructs then were electroporated into *E. chrysanthemi* 3937 (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 gene knockouts. Mutations in the resultant kanamycin-resistant and ampicillin-sensitive colonies then were confirmed with isolated DNA by PCR using transposon and target-gene-specific primers.

Virulence assays were conducted on African violet (var. Katja) leaves by the methods described in (Yang et al. 2002). Rates of lesion spreading by mutant *E. chrysanthemi* 3937 strains were compared with the wild type in at least four different assays to confirm results.

LITERATURE CITED

Boch, J., Joardar, V., Gao, L., Robertson, T. L., Lim, M., and Kunkel, B. N. Identification of *Pseudomonas syringae* pv. *tomato* genes induced during infection of *Arabidopsis thaliana*. Mol. Microbiol. In press.

Brickman, T. J., Barry, C. E., and Hackstadt, T. 1993. Molecular cloning and expression of *hctB* encoding a strain-variant chlamydial histone-like protein with DNA-binding activity. J. Bacteriol. 175:4274-4281.

Burr, T. J., and Schroth M. N. 1977. Occurrence of soft-rot *Erwinia* spp. in soil and plant material. Phytopathology 67:1382-1387.

Calhoun, D. H., Bonner, C. A., Gu, W., Xie, G., and Jensen, R. A. 2001. The emerging periplasm-localized subclass of AroQ chorismate mutases, exemplified by those from *Salmonella typhimurium* and *Pseudomonas aeruginosa*. Genome Biol. 2:research0030.1-0030.16.

Chatterjee, A. K., Dumenyo, C. K., Liu, Y., and Chatterjee, A. 2000. *Erwinia*: Genetics of pathogenicity factors. Pages 236-259 in: Encyclopedia of Microbiology, Vol. 2. J. Lederberg, ed. Academic Press, New York.

Cother, E. J., and Gilbert, R. L. 1990. Presence of *Erwinia chrysanthemi* in two major river systems and their alpine sources in Australia. J. Appl. Bacteriol. 69:629-738.

Cui, Y., Mukherjee, A., Dumenyo, C. K., Liu, Y., and Chatterjee, A. K. 1999. *rsmC* of the soft-rotting bacterium *Erwinia carotovora* subsp. *carotovora* negatively controls extracellular enzyme and harpin (Ecc) production and virulence by modulating levels of regulatory RNA (*rsmB*) and RNA-binding protein (RsmA). J. Bacteriol. 181:6042-6052.

Danchin, A., and Sekowska, A. 2000. Expression profiling in reference bacteria: Dreams and reality. Genome Biol. 1:1024.1-1024.5.

DeLisa, M. P., Wu, C.-F., Wang, L. Valdes, J. J., and Bentley, W. E. 2001. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. J. Bacteriol. 183:5239-5247.

Expert, D. 1999. Withholding and exchanging iron: Interactions between *Erwinia* spp. and their plant hosts. Annu. Rev. Phytopathol. 37:307-334.

Franza, T., and Expert, D. 1991. The virulence-associated chrysobactin iron uptake system of *Erwinia chrysanthemi* 3937 involves an operon encoding transport and biosynthetic functions. J. Bacteriol. 173:6874-6881.

Gomes, C. M., Silva, G., Oliveira, S., LeGall, J., Liu, M.-Y., Xavier, A. V., Rodrigues-Pousada, C., and Teixeira, M. 1997. Studies on the redox centers of the terminal oxidase from *Desulfovibrio gigas* and evidence for its interaction with rubredoxin. J. Biol. Chem. 272:22502-22508.

Gonzalez-Pastor, J. E., San Millan, J. L., Castilla, M. A., and Moreno, F. 1995. Structure and organization of plasmid genes required to produce the translation inhibitor microcin C7. J. Bacteriol. 177:7131-7140.

Gross, D.C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Annu. Rev. Phytopathol. 29:247-278.

Hassouni, M. E., Chambost, J. P., Expert, D., Van Gijsegem, F., and Barras, F. 1999. The minimal gene set member *msrA*, encoding peptide methionine sulfoxide reductase, is a virulence determinant of the plant pathogen *Erwinia chrysanthemi*. Proc. Natl. Acad. Sci. U.S.A. 96:887-892.

Haygood, R. A., Strider, D. L., and Echandi, E. 1982. Survival of *Erwinia chrysanthemi* in association with *Philodendron selloum*, other greenhouse ornamentals, and in potting media. Phytopathology 72:853-859.

Kammler, M., Schon, C., and Hantke, K. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J. Bacteriol. 175:6212-6219.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II, and Peterson, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175-176.

López-Solanilla, E., Llama-Palacios, A., Collmer, A., García-Olmedo, F., and Rodríguez-Palenzuela, P. 2001. Relative effects on virulence of mutations in the *sap*, *pel*, and *hrp* loci of *Erwinia chrysanthemi*. Mol. Plant-Microbe Interact. 14:386-393.

Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras F. 2001. SoxR-dependent response to oxidative stress and virulence of *Erwinia*

chrysanthemi: The key role of SufC, an orphan ABC ATPase. Mol. Microbiol. 39:960-972.

Nakamura, M., Saeki, K., and Takahashi, Y. 1999. Hyperproduction of recombinant ferredoxins in *Escherichia coli* by coexpression of the ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster. J. Biochem. 126:10-18.

Perna, N., Plunkett, G., III, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Posfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A., and Blattner, F. R. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157: H7. Nature 409:529-533.

Perombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot *Erwinias*. Annu. Rev. Phytopathol. 18:361-387.

Ried, J. L., and Collmer, A. 1987. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. Gene 57:239-246.

Saier, M. H., and Reizer, J. 1994. The bacterial phosphotransferase system: New frontiers 30 years later. Mol. Microbiol. 13:755-764.

Smith, H. 2000. State and future of studies on bacterial pathogenicity: Impact of new methods of studying bacterial behavior in vivo. Pages 265-282 in: Virulence Mechanisms of Bacterial Pathogens, 3rd ed. K. Brogden, J. Roth, T. Stanton, C. Bolin, C. Minion, and M. Wannemuehler, eds. American Society for Microbiology Press, Washington, D.C.

Stanghellini, M. E. 1982. Soft-rotting bacteria in the rhizosphere. Pages 249-261 in: Phytopathogenic Prokaryotes, Vol. 1. M. Mount and G.

Lacy, eds. Academic Press, New York.

van den Berg, W. A., Hagen, W. R., and van Dongen, W. M. 2000. The hybrid-cluster protein ('prismane protein') from *Escherichia coli*. Characterization of the hybrid-cluster protein, redox properties of the [2Fe-2S] and [4Fe-2S-2O] clusters and identification of an associated NADH oxidoreductase containing FAD and [2Fe-2S]. Eur. J. Biochem. 27:666-676.

Vásquez, C. C., Saavedra, C. P., Loyola, C. A., Araya, M. A., and Pichuantes S. 2001. The product of the *cysK* gene of *Bacillus stearothermophilus* V mediates potassium tellurite resistance in *Escherichia coli*. Curr. Microbiol. 43:418-423.

Vayda, M. E., Antonov, L. S., Yang, Z., Butler, W. O., and Lacy, G. H. 1992. Hypoxic stress inhibits aerobic wound-induced resistance and activates hypoxic resistance to bacterial soft rot. Am. Potato J. 69:239-253.

Wassenaar, T. M., and Gaastra, W. 2001. Bacterial virulence: Can we draw the line? FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 201:1-7.

Wei, Y., Lee, J.-M., Richmond, C., Blattner, F. R., Rafalski, J. A., and LaRossa, R. A. 2001. High-density microarray-mediated gene expression profiling of *Escherichia coli*. J. Bacteriol. 183:545-556.

Yang, C.-H., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., Chen, J. W.-T., Perna, N. T., and Keen, N. T. 2002. *hrp* genes of *Erwinia chrysanthemi* 3937 are important virulence factors. Mol. Plant-Microbe Interact. 25:472-480.

AUTHOR-RECOMMENDED INTERNET RESOURCE

The Institute for Genomic Research database:
www.tigr.org/tdb/mdb/mdbinprogress.html