JOURNAL OF BACTERIOLOGY, June 1988, p. 2419–2426
0021-9193/88/062419-08$02.00/0

Vol. 170, No. 6

Systemic Virulence of *Erwinia chrysanthemi* 3937 Requires a Functional Iron Assimilation System

CORINE ENARD, ANNICK DIOLEZ, AND DOMINIQUE EXPERT*

Laboratoire de Pathologie Végétale, Institut National Agronomique Paris-Grignon, 75231 Paris Cedex 05, France

Received 24 November 1987/Accepted 22 February 1988

In *Erwinia chrysanthemi*, conditions of iron starvation initiate production of a catechol-type siderophore and enhance production of three outer membrane polypeptides. Twenty-two mutants affected in the different stages of this iron assimilation system were isolated by mini-Mu insertion mutagenesis. All of them failed to induce systemic soft rot on axenically grown *Saintpaulia* plants. From the siderophore auxotrophs and the iron uptake mutants, clones having recovered the missing function(s) were isolated by using the in vivo cloning vector pULB113 (RP4::mini-Mu). An R-prime plasmid containing a ca. 35.5-kilobase-pair DNA insert was identified. Restoration of the iron functions restored partially, if not completely, the virulence of the parental strain.

Erwinia chrysanthemi, like other pectinolytic *Erwinia* spp., produces soft rot diseases on a wide range of plants in tropical and subtropical countries. Also, plants grown in greenhouses in temperate areas exhibit soft rot symptoms characteristic of pectinolytic erwinias. These bacteria enter the plant, usually through wounds, and then multiply and spread in the intercellular space. This process is largely facilitated by bacterial pectolytic enzymes that attack cell walls and result in cell death (10, 21; M. Boccara, A. Diolez, M. Rouve, and A. Kotoujansky, Physiol. Mol. Plant Pathol., in press). Unlike most of the soft rot erwinias, *E. chrysanthemi* gives rise to a systemic disease (32). Systemic disease symptoms occur when the bacteria express virulence upon spreading through the vascular system of the plant. Besides secretion of pectin-degrading enzymes, which is a function essential for induction of maceration symptoms, several outer membrane components are of importance for the virulence of *E. chrysanthemi*, as was shown with strain 3937, which specifically infects *Saintpaulia* plants. Through a study of mutants resistant to bacteriocins, Expert and Toussaint (13) suggested that the inability to acquire iron may attenuate the virulence of these bacteria. In these mutants, the loss of virulence was correlated with modifications in the pattern of three outer membrane polypeptides migrating in the 80,000-dalton range, the production of which appeared to be greatly enhanced in wild-type cells grown under iron starvation. These mutants were also found to lack the O-antigenic part of their lipopolysaccharide molecule, but further studies have demonstrated that mutants missing only this structure remained completely virulent (14, 30).

Iron is essential for living cells, and competition for iron between host and microbe is a mechanism that may determine the outcome of an invasion of a vertebrate host by some pathogenic bacteria. During infection, mammalian hosts react by releasing the high-affinity iron-binding proteins tranferrin (serum) and lactoferrin (secretions) in the infected area. This results in withholding iron from invading pathogens, and the ability of the host to sequester iron was called nutritional immunity (6, 36). In response to iron limitation, microorganisms produce low-molecular-weight, high-affinity iron-chelating agents, termed siderophores, and the components required for specific uptake of the ferric complex of the siderophore (for a review, see reference 37).

* Corresponding author.

The role of this metal in the infection of plants by phytopathogens is not understood. A survey of gram-negative and fungal phytopathogens showed that although they all produced siderophores, no correlation between siderophore activity and infectivity could be established (26). A recent study of *Pseudomonas syringae* revealed that Fe(III) uptake did not appear to have a determinative role in the virulence of the bacterium when tested on cherry fruit (9). Another study, however, showed that the iron level in potato plants can influence symptom expression of the pathogenic fungus *Verticillium dahliae* (2).

Here, we provide evidence that iron acquisition acts as a virulence factor in *E. chrysanthemi* 3937. The production of low-iron-regulated outer membrane proteins was shown to be correlated with the production of a catechol-type siderophore. A series of mutants defective either in production of the catechol compounds or in utilization of the exogenous ferric complex were isolated and characterized. All of them appeared to have reduced virulence. From the siderophore auxotrophs, we isolated transconjugants which had recovered the missing function(s) by using the in vivo cloning vector pULB113 (RP4::Mu3A) (35). This vector carries a mini-Mu with a functional transposase and can spontaneously integrate in its host chromosome by replicon fusion. Upon mini-Mu-mediated excision, R-prime plasmids which carry random pieces of the host chromosome are generated. In the two cases studied so far, return of the iron-related function partially restored virulence on *Saintpaulia* plants.

MATERIALS AND METHODS

Strains and media. The bacteriophages, plasmids, and strains used in this work are described in Table 1. Rich medium was L broth (24), and minimal medium M63 (24) contained 2 g of glucose per liter. The M63 medium was deferrated as described previously (13). Media were made iron starved by addition of EDDA [ethylenediamine-*N*,*N*'-bis(2-hydroxyphenylacetic acid); 100 μg/ml] (Fluka AG Chemical Co.), a strong nonutilizable iron chelator. An EDDA stock solution was deferrated by the procedure of Rogers (29). For enzyme assays, M9 medium (24) was supplemented with 5 g of glycerol and 1 g of yeast extract per liter. Five grams of sodium polygalacturonate (Sigma Chemical Co.) and 5 g of carboxymethylcellulose (Serlabo Co.) were added per liter for polygalacturonate lyase and carboxymethylcellulase activities, respectively.

TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, bacteriocin, or plasmid	Characteristics[a]	Source (reference)
E. chrysanthemi		
3937	Wild type, isolated from *Saintpaulia ionantha*	Kotoujansky et al. (20)
B374	Wild type	Hamon and Peron (16)
PMV 4078–PMV 4081	Mu dII1734 lysogenic derivatives of 3937, catechol nonproducers	This work
PMV 4082–PMV 4084	Mu dII1734 lysogenic derivatives of 3937, catechol nonproducers, also affected in transport	This work
PMV 4085–PMV 4089	Mu dII1734 lysogenic derivatives of 3937; affected in late step of siderophore biosynthesis?	This work
PMV 4090	Mu dII1734 lysogenic derivative of 3937, unable to grow on EDDA-L agar medium	This work
PMV 4091–PMV 4097	Mu dII1734 lysogenic derivatives of 3937; affected in excretion of siderophore?	This work
PMV 4098–PMV 4099	Mu dII1734 lysogenic derivatives of 3937, affected in transport	This work
PMV 4100	Spontaneous mutant of 3937, Phe$^-$ Trp$^-$ Tyr$^-$ PABA$^-$ (Aro$^-$)	This work
E. coli		
POII1734	F$^-$ *araD139 ara*::(Mu cts)*3Δ(lac)X74 galU galK rpsL* Mu dII1734	Castilho et al. (7)
RW193	F$^-$ *thi entA proC leuB trpE tsx lacY rpsL galK ara mtl xyl azi supE44*	Received from A. Pugsley
AN93	F$^-$ *proC leuB trpE purE thi lacY rpsL entE fhuA tsx supA azi mtl xyl*	Received from J. B. Neilands
Bacteriophages		
Mu *d*II1734	Mu *c*ts62 Δ(Mu *A B*) Km^r Δ(*lac'ZYA*)	Castilho et al. (7)
Mu *c*ts62	Thermoinducible	M. Howe (17)
PhiEC-2	Wild type	Résibois et al. (28)
GU5	IncP plasmid-specific phage	Barth et al. (3)
Bacteriocins[b]		
1277, 1455, 1456, 1500, 1521, 1871, 1884, and 3912	Mitomycin C-inducible bacteriocins isolated from *Erwinia* strains	Expert and Toussaint (13)
20D3		Thiry-Braipson et al. (34)
Plasmids		
pULB113	RP4::Mu3A, Tc^r Ap^r Km^r	Van Gijsegem and Toussaint (35)
pSup2021	(pBR325-Mob)::Tn*5*	Simon et al. (31)

[a] Phe, Phenylalanine; Trp, tryptophan; Tyr, tyrosine; PABA, *para*-aminobenzoic acid.
[b] Each number refers to a bacteriocin isolated or used previously (13) and corresponds to the bacteriocin-producing strain number.

When required, the media were solidified by using Difco agar (12 or 15 g/liter for plates and 7 g/liter for soft overlays). When necessary, the following antibacterial agents were added to the media: 20 μg of kanamycin (Km), ampicillin (Ap), or tetracycline (Tc) or 100 μg of streptomycin (Sm) per ml; 1 g of sodium deoxycholate (DOC; Sigma), EDTA (Sigma), or sodium dodecyl sulfate (SDS) (Serlabo Co.) per liter; or 10 g of Triton X-100 (Bio-Rad Laboratories) per liter. Amino acids and 2,3-dihydroxybenzoic acid (DHBA) (Sigma Chemical Co.) and vitamins were added at final concentrations of 40 and 1 μg/ml, respectively. Except when otherwise specified, all incubations were carried out at 30°C. Glassware for deferrated M63 medium was treated as described by Pugsley and Reeves (27).

Isolation of mutants. A multiple aromatic biosynthetic mutant was isolated by three repeated ampicillin treatments (24). Mu-mediated mutagenesis was performed by infecting an L broth culture of strain 3937 growing exponentially (optical density at 600 nm [OD_{600}] about 0.6) with a lysate of phage Mu (10^8 PFU/ml) prepared by heat induction of strain POII1734 (7) in a final volume of 0.4 ml. The multiplicity of infection (MOI) was 0.3. The suspension was incubated at 30°C for 20 min and then plated onto L agar medium supplemented with kanamycin. The frequency of Km^r clones (relative to the total number of Mu *c*ts62 phage particles used for infection) was 10^{-4}. Kanamycin-resistant survivors were replicated onto EDDA-L agar medium.

Heat induction of *E. chrysanthemi* Mu *c*ts lysogens. The presence of a Mu *c*ts prophage in *E. chrysanthemi* was scored at 37°C by the appearance of a halo of lysis surrounding the clones to be tested, these having been previously picked onto a soft agar overlay seeded with the indicator strain B374.

Sensitivity. Sensitivity to bacteriocins, bacteriophages, or membrane-active agents was determined as described previously (13).

Cross-feeding assay. Plates were poured with a 25-ml volume of EDDA-L agar medium seeded with an overnight L broth culture of the indicator strain at a final concentration of 10^4 CFU/ml. After cooling, the medium was spotted with 10 μl of a suspension of the mutant to be tested in soft agar medium containing EDDA (10 μg/ml). Such a concentration of chelator in the medium allowed the mutant strain to be induced for the iron system but did not inhibit its growth. The presence of a halo of growth surrounding the strain being tested indicated its ability to cross-feed the indicator strain.

Matings. Donors harboring plasmid pULB113 or their derivatives were mated with recipient strains by the method of Van Gijsegem and Toussaint (35).

Chemical assays for detecting siderophores. Catechol was assayed by the method of Arnow (1), and hydroxamate was assayed by the methods of Csáky (11) or Leong and Neilands (22).

Polygalacturonate lyase and endoglucanase activities. The detection of polygalacturonate lyase and endoglucanase activities in supernatant fluids of bacterial cultures was performed in polygalacturonate or carboxymethylcellulose-containing medium by the cup-plate technique. This method is based on radial diffusion of the enzymes into a substrate-

bearing agar gel as described by Bertheau et al. (4). Polygalacturonate lyase isoenzymes were detected by electrofocusing in ultrathin polyacrylamide gels, also by the procedure of Bertheau et al. (4).

Outer membrane preparation and analysis. Triton-insoluble walls (outer membranes) were prepared and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (13).

DNA methods. Total DNA was isolated from bacterial cells by the method of Klötz and Zimm (19) and plasmid DNA was prepared by the clear lysate method (8). Density equilibrium centrifugation was carried out in a Centrikon T-2070 ultracentrifuge (Kontron Inst.) at 50,000 rpm with a VTi 65 vertical rotor (Beckman). Miniscale plasmid DNA analysis was performed by the procedure of Kado and Liu (18). Total bacterial DNA preparations were hydrolyzed with the restriction endonuclease *Bam*HI (Boehringer Mannheim), and plasmid DNA preparations with the endonuclease *Pst*I (Pharmacia) as described by Maniatis et al. (23). The DNA digests were analyzed by horizontal agarose (0.8%) gel electrophoresis in Tris-borate-EDTA buffer (23). DNA-DNA hybridization analysis was carried out by the method of Southern (33). Plasmid DNA probe was labeled by nick translation with [α-^{32}P]dCTP (600 Ci/mmol) according to the manufacturer's specifications (Amersham Inc.).

Pathogenicity tests. The plant tissue maceration assay was described previously (13).

The whole-plant assay was carried out on *Saintpaulia* plants (African violets) axenically cultured in test tubes, regenerated from *Saintpaulia ionantha* cv. Blue Rhapsody, as described by Bilkey et al. (5) with some modifications. Shoots were regenerated from leaf disks. Benzyladenine was added in the shoot induction medium at a concentration of 0.1 mg/liter instead of 0.01 mg/ml. In the root induction medium, mineral salts were diluted twofold and no hormone was added. Test tube-grown plants were grown at 24 to 27°C for 16 h under illumination with 2,500 Lux, followed by 8 h in the dark.

When the plant was about 2 months old, one leaf was scratched with a Pipetman tip (Gilson Co.), and the wounded part was infected with 5 μl of a bacterial suspension in a 0.15 M NaCl solution, at a concentration of 3×10^8 to 5×10^8 CFU/ml. To check the phenotype of bacteria present in planta, pieces of necrotic tissue were suspended and stirred in 1 ml of 0.01 M $MgSO_4$. The bacterial suspension obtained was counted on L agar medium supplemented with the appropriate antibiotic.

RESULTS

Identification of catechol-dependent iron uptake system in *E. chrysanthemi*. When grown under conditions leading to iron starvation, in deferrated M63 or EDDA-M63 medium, strain 3937 produced extracellular catechol, but no hydroxamate was found. To check whether this catechol was derived from DHBA, the common intermediate in the biosynthetic pathway of most catechol-type siderophores (25), we isolated a multiple aromatic auxotroph, altered in the pathway before chorismate, this compound being a precursor of DHBA and the branch point intermediate in the aromatic amino acid biosynthetic pathway (38). As expected, this strain failed to produce catechol and grew poorly when plated onto EDDA-L agar medium. Its growth was reversed with addition of the synthetic product DHBA. Such a mutant that was cross-fed with the parental strain also responded to cross-feeding with the *Escherichia coli entA* (RW193) or *entE* (AN93) derivative, illustrating that it can utilize a precursor of DHBA or DHBA itself as a growth factor. On the other hand, it gave rise to a weak halo of growth when cross-fed with an *E. coli* enterobactin-producing strain, and conversely, the *E. coli entA* mutant could not be cross-fed with the *E. chrysanthemi* wild-type strain.

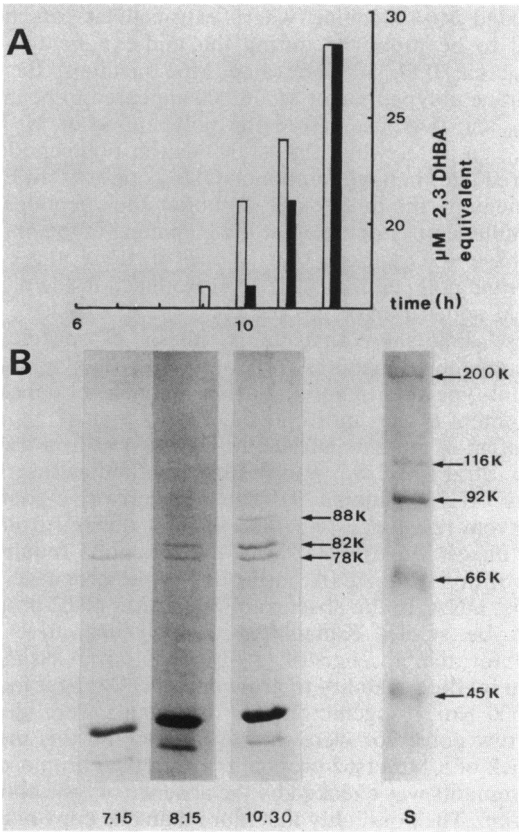

FIG. 1. Production of a catechol-type siderophore and overproduction of three outer membrane proteins in strain 3937 grown under iron starvation. At various stages of bacterial growth in EDDA-M63 medium (A, open columns) and in deferrated M63 medium (A, solid columns), catechol was assayed with DHBA as the standard. Protein composition of the Triton-insoluble envelope fraction (outer membrane) was analyzed by SDS-PAGE in 12% acrylamide with 20- to 40-μg protein samples prepared at different times of growth (shown in hours below each lane) in deferrated M63 medium (B). The apparent molecular sizes of standard proteins (lane S) are indicated in kilodaltons (K).

The growth cycle of wild-type cells was examined under these conditions of iron nutrition (Fig. 1). Under normal conditions, i.e., in deferrated M63 medium supplemented with 2 μM Fe(II), the doubling time was about 70 min, while it reached about 90 min in deferrated M63 as well as in EDDA-M63 medium. However, in EDDA-M63 medium, the lag period was longer and the final absorbance was lower than in deferrated M63, suggesting that iron starvation must be more acute in a medium where more available iron was chelated. Unlike the parental strain, the multiple aromatic auxotroph was shown to grow poorly under iron starvation.

We looked for the presence of catechol in supernatant fluids and for the level of the polypeptides of molecular weights 78,000, 82,000, and 88,000 in cell outer membranes at various stages of the growth of strain 3937 (Fig. 1). In EDDA-M63 medium, catechol was detected at the beginning of the exponential phase (OD_{600}, ca. 0.4), i.e., earlier than in

deferrated M63 medium, where extracellular catechol appeared to be produced during the mid-exponential phase (OD_{600}, ca. 0.8). In deferrated M63 medium, the outer membrane polypeptide of M_r 78,000 appeared to be induced (OD_{600}, ca. 0.4) just before the polypeptide of M_r 82,000 (OD_{600}, ca. 0.5), while the 88,000-dalton polypeptide only appeared later in larger amounts (OD_{600}, ca. 0.9). In EDDA-M63 medium, the three outer membrane polypeptides could be simultaneously detected at the beginning of the exponential phase (OD_{600}, ca. 0.2). None of these three outer membrane polypeptides were overproduced in the presence of 2 μM added Fe(II).

These data show that the synthesis of specific outer membrane components is correlated with the synthesis of a catechol-type siderophore and that this control depends on iron content in the culture medium.

Isolation of mutants affected in the catechol iron transport system. Strain 3937 was mutagenized by random insertion of the Mu dII1734 genome (7). This Mu derivative contains a kanamycin resistance selectable marker derived from Tn*5*. Since phage Mu dII1734 lacks the functions required for transposition of its DNA, insertions of the prophage within the host DNA, in the absence of helper Mu *c*ts62 prophage, should be stable. Kanamycin-resistant derivatives were tested for the absence of a functional iron assimilation system by their inability to grow on EDDA-L agar medium. Of 5,000 Km^r lysogenic clones, 26 mutants were identified that grew poorly or were unable to grow on this medium. The lack of a Mu *c*ts62 prophage within the chromosome of these mutants was checked by the absence of lysis upon heat induction. The possibility that more than one copy of the Mu dII1734 genome was present within the host DNA was examined by Southern blot hybridization of the *Bam*HI total DNA digest of the mutants, with the Tn*5*-containing plasmid pSup2021 (31) as the ^{32}P-labeled probe (data not shown). Of the 26 mutants selected, 22 contained a single insertion of the Mu dII1734 genome, whereas the four other contained two insertions. Only the single-insertion mutants were analyzed further.

Characterization of the mutants. All the mutants grew normally in M63 minimal medium, ruling out the possibility of having selected an aromatic auxotroph. The mutants were first characterized on the basis of their ability to produce DHBA-containing compounds by the method of Arnow and to utilize the ferric complex of the siderophore in cross-feeding experiments with the parental strain (Fig. 2). Seven mutants failed to produce DHBA-containing compounds and were designated siderophore auxotrophs. Therefore, they should be blocked before DHBA at one of the three steps in the biosynthetic pathway from chorismate. Three mutants of the seven siderophore auxotrophs and 2 mutants of the 15 remaining DHBA-producing mutants could not be cross-fed with the parental strain. Such mutants are likely to be defective in the transport of the externally formed ferric complex or, possibly, in removal of iron from the complex.

Three classes of mutants might be considered: (i) class I includes four siderophore auxotrophs, (ii) class II includes three mutants altered both in biosynthesis of the siderophore and in utilization of the ferric complex of the siderophore, and (iii) class III contains two mutants defective only in utilization of the ferric complex (Tables 1 and 2). Additional evidence favoring the existence of these three classes are given by cross-feeding assays. The mutants belonging to class I and II were able to cross-feed the multiple aromatic auxotroph, giving more or less extensive halos of growth according to the mutant tested, and this suggests that these

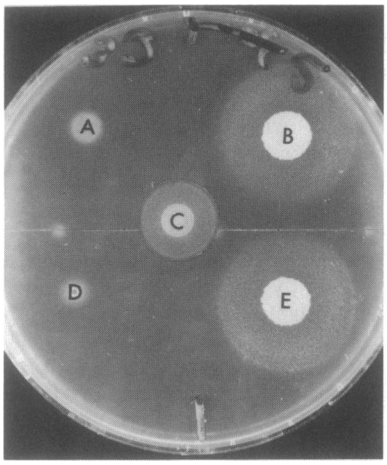

FIG. 2. Cross-feeding assay. For details, see Materials and Methods. The indicator strain used in this assay was the class I mutant PMV 4078. (A and D) response (no halo of growth) given by a mutant of class I (PMV 4079) and a mutant of class II (PMV 4082), respectively. (C) Wild-type strain (halo of growth). (B and E) Transconjugants PMV 4079(R'4) and 4082(R'4), respectively (big halos).

mutants produced a precursor of DHBA that acts as a growth factor. However, the mutants of class I failed to grow on EDDA-L agar medium supplemented with DHBA as a growth factor. The two mutants of class III gave rise to halos more extensive than with the wild-type strain. Such a catechol-hyperproductive phenotype suggests the absence of internalization of the ferric complex of the siderophore; these mutants also cross-fed the mutants of class I but not the mutants of class II.

The remaining DHBA-producing mutants that were able to utilize the exogenous complex of the siderophore could be divided into two additional groups: five of them (class IV) grew poorly on EDDA-L agar medium and could cross-feed the mutants of class I but not the mutants of classes II and III, and the others (class V) were unable to cross-feed any mutants. The mutants belonging to class IV might liberate a functional ligand, less efficient than the final product, that is recognized by the transport system. The mutants of class V might be altered in excretion of the siderophore rather than in a late step of biosynthesis. Finally, one mutant displayed an intermediate phenotype between the siderophore auxotrophs and the wild-type strain.

TABLE 2. Different classes of mutants

Class	Phenotype					
	Catechol production[a]	Cross-fed by wild type[b]	Ability to cross-feed class[b]:			Low-iron-regulated proteins (kDa)
			I	II	III	
I	<10	+	−	−	−	78, 82, 88
II	<10	−	−	−	−	78, 88
III	50–60	−	++	−	−	78, 82, 88
IV	50–60	+	+	−	−	78, 82, 88
V	50–60	+	−	−	−	78, 82, 88
Wild type	25–30	ND	+	−	−	78, 82, 88

[a] Production of catechol is given in micromolar DHBA equivalents.

[b] Symbols: −, no halo of growth; +, halo of growth present when cross-fed with wild-type strain; ++, large halo of growth; ND, not done.

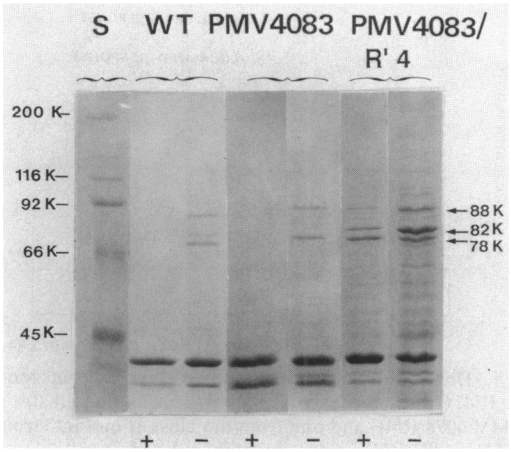

FIG. 3. Protein composition of the Triton-insoluble envelope fraction (outer membrane) prepared from wild-type strain 3937 (WT), a mutant of class II, PMV 4083, and its transconjugant PMV 4083(R'4) at low and high iron levels. SDS-PAGE was carried out on samples prepared from 5 to 10 ml of culture in M63 medium (2 μM Fe in lanes labeled +) or in deferrated M63 medium (lanes labeled −). The apparent molecular sizes of standard proteins (lane S) and of low-iron-inducible proteins are referred to as in Fig. 1B. The two other class II mutants (not shown) gave the same pattern of PMV 4083.

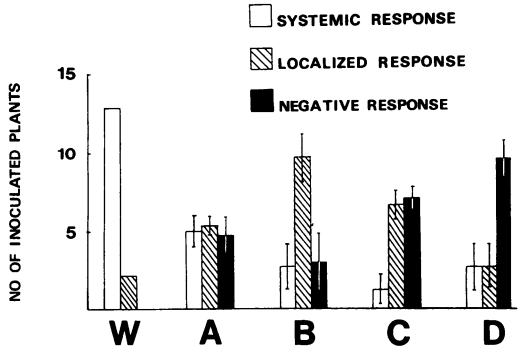

FIG. 4. Groups of mutants differentiated by distribution of their responses on plants. Each mutant was inoculated to 15 plants as described in Materials and Methods. According to the distribution of the three types of responses, systemic, localized, and negative, as defined in Materials and Methods and in the text, the mutants fell into four statistically significant groups (A, B, C, and D). A chi-square test revealed homogeneity within each group ($P = 0.1$). W, Wild-type strain.

The profiles of outer membrane proteins from the parental strain and all the mutants grown in low-iron medium were compared. The three iron-regulated outer membrane polypeptides appeared normal (data not shown), except in the three mutants of class II. The class II mutants lacked the 82,000-molecular-weight polypeptide (Fig. 3). The 82,000-molecular-weight polypeptide might be the outer membrane receptor for the ferric complex of the siderophore.

Finally, no modification in the envelope structure of these mutants was evident in their sensitivity to the bacteriocins described previously (13), to phage PhiEC-2 (30), or to various membrane-active agents such as DOC, SDS, Triton X-100, and EDTA, that was similar to that of the parental strain. Phenotypes of the mutants are summarized in Table 2.

Pathogenicity of the mutants. The 22 mutants analyzed in this study produced normal amounts of extracellular pectate lyases and endoglucanases compared with the parental strain (data not shown). For testing pathogenicity, an assay was devised on axenically cultured *Saintpaulia* plants. When tested on such plants under the growth conditions described in Materials and Methods, the wild-type strain caused in most cases a systemic response. After inoculation, an oily translucent patch was evident at the inoculated site within 12 to 24 h, and this patch subsequently became brown. In 48 to 72 h, the maceration had spread all over the inoculated leaf and its petiole and was progressing towards the other aerial parts of the plant. In 6 to 8 days the plant had completely collapsed. Unlike the parental strain, which gave rise to more than 80% systemic responses (Fig. 4), all the mutant strains displayed a similar behavior: they were unable to express virulence upon spreading through the vascular system. When each of them was tested on 15 plants, two main phenotypes were observed: they gave rise either to (i) local maceration which did not progress beyond the petiole of the inoculated leaf (noted as localized response in Fig. 4) or (ii) an absence of maceration (negative or necrotic response, as given in Fig. 4). For all the mutants, a delay of 1 or 2 days was observed when they remained able to induce symptoms. In summary (Fig. 4), the mutants can be classified in four groups with respect to the frequency of the three different responses described above. There was no correlation with respect to the siderophore auxotrophy phenotype and the particular group a mutant fell into.

In preliminary experiments, we observed that the axenically grown plants responded like plants grown in a greenhouse when infected with the wild-type strain and with nonpathogenic mutants. Interestingly, the axenically grown plants responded to infection with strain 3937 in about 7 days. The time required for infection to lead to the destruction of normal plants in greenhouse tests was 1 month or more.

In vivo cloning of the genes involved in siderophore production and transport. The closely related *E. coli* and *E. chrysanthemi* 3937 may have similar functions required for the biosynthesis of siderophore precursors, particularly in the conversion of chorismate into DHBA. We thought it might be possible to complement an *E. coli entA* mutant with a corresponding *entA*-like gene from strain 3937 by using the pULB113 (RP4::Mu3A) plasmid.

Therefore, strain 3937 carrying pULB113 was mated with the *E. coli entA* strain RW193 Sm^r. *E. coli* transconjugants, expected to have recovered the ability to produce enterobactin, were screened on EDDA-L agar medium, the donor being counterselected with streptomycin. Transconjugants able to grow on this medium occurred at a frequency of about 10^{-7}. Several clones suspected to be complemented were isolated, and one of them was further characterized. This clone was able to produce DHBA-containing compounds and to cross-feed the parental strain RW193 when grown in iron-depleted medium. This suggested that the bacteria had recovered the ability to synthesize enterobactin, probably because they had received an allele that can function as *entA* from *E. chrysanthemi*, as a result of gene transfer by R-prime formation. The complemented strain received the pULB113 plasmid since it was Km^r Ap^r Tet^r and sensitive to phage GU5. In addition, plasmid-cured derivatives isolated from this clone as being resistant to phage GU5 appeared to have simultaneously lost the *entA*-like donor marker.

The covalently closed circular DNA content of the complemented clone was isolated and hydrolyzed with the

restriction endonuclease *Pst*I. We found the eight *Pst*I fragments that correspond to the pULB113 vector. Also, an additional 2.9-kilobase (kb) fragment that appeared to be a doublet and four additional fragments were evident. The 2.9-kb doublet corresponds to internal fragments of mini-Mu. This is consistent with the presence of two copies of the mini-Mu in the same orientation flanking the bacterial cloned DNA, as previously described in R-prime formation (35). The size of the chromosomal DNA insert was about 35.5 kb.

To determine whether the cloned DNA sequence carried on the R-prime plasmid, designated R'4, could restore siderophore production in the *E. chrysanthemi* mutants of class I and class II, R'4 was transferred into each of the mutants by conjugation. In a first step, transconjugants having acquired the RP4 plasmid markers were selected on a medium containing tetracycline, ampicillin, and kanamycin, the donor being counterselected by prototrophy. These appeared at a frequency of 10^{-3}, and 50 to 90% of them, according to the recipient strain, appeared to be able to grow on EDDA-L agar medium. For each recipient strain, transconjugants having acquired the new trait were streaked twice on M63 minimal agar medium supplemented with tetracycline for single-colony isolation. Colonies having lost the ability to grow on EDDA-L agar medium occurred because of the instability of R-prime plasmids in Rec^+ recipient cells (35).

The mutants that had received R'4 were able to produce catechol and to cross-feed the multiple aromatic auxotroph as well as the class I mutants when cultured on iron-depleted medium (Fig. 2). Several of the transconjugants appeared to overproduce catechol, even under high-iron conditions. As shown in Fig. 3, the mutants of class II which lacked the 82,000-molecular-weight polypeptide recovered the ability to produce this polypeptide; they also appeared to express the three outer membrane proteins constitutively. Analysis of the plasmid content of these clones revealed the absence of the initially transferred R'4 plasmid, although they still expressed the RP4 antibiotic resistance markers. In some cases, a plasmid corresponding to pULB113 could be detected (data not shown). This is expected since genetic rearrangements frequently occur when homospecific R-prime plasmids are transferred into Rec^+ cells. When one of the transconjugants was mated with the *E. coli entA* recipient strain, the *E. chrysanthemi entA* allele carried on plasmid R'4 could not be cotransferred with the antibiotic resistance markers of the vector. This indicated that homologous recombination between chromosomal and plasmid DNA had occurred. The constitutive phenotype of the transconjugants might be due to such recombination events.

One clone isolated from a mutant of class I (PMV 4078) and one isolated from a mutant of class II (PMV 4083) having recovered the missing function(s) were tested for virulence on axenically grown *Saintpaulia* plants. They gave rise to a phenotype intermediate between that of the wild-type response and the siderophore auxotroph mutants. For both of them, maceration appeared 24 h after inoculation in all plants, as with the wild-type strain. The transconjugants of class II could, in all cases, macerate at least the inoculated leaf with its petiole (localized response), while the initial mutant strain was mostly unable to induce any symptoms (Fig. 5); such a difference could be detected to a lesser extent between the transconjugant and the initial mutant of class I. However, generalization of the disease was only observed in a few cases. Such a phenotype with regard to virulence might be the consequence of the instability of the transconjugants in planta. This was likely to be the case, since the R-prime appeared to be lost from the transconjugants with no selection. Bacteria reisolated from infected plants were all able to grow on EDDA-L agar medium and all were kanamycin and ampicillin resistant. This suggests that complementing sequences provided the mutant strains with genetic functions which are maintained for growth in planta.

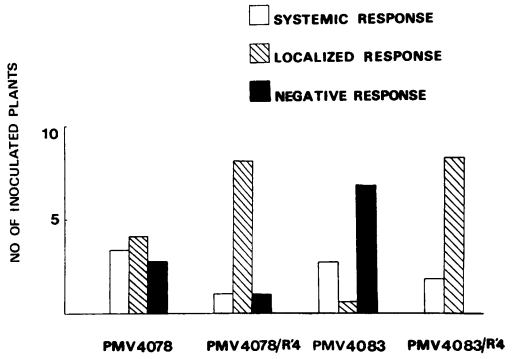

FIG. 5. Distribution of plant responses given by transconjugants carrying R'4. One clone deriving from the class I mutant strain PMV 4078 [PMV 4078(R'4)] and one from the class II mutant strain PMV 4083 [PMV 4083(R'4)] were each inoculated to 10 plants. The parental mutant strains are also shown as a control. For further details, see Fig. 3 legend and the text.

DISCUSSION

The possible role of an iron assimilation system in the pathogenicity of *E. chrysanthemi* 3937 was investigated. We determined the importance of the production of the siderophore and the functions involved in iron assimilation and in virulence. We showed that, in this strain, iron starvation initiates the production of a catechol-type siderophore. Although the structure of the biologically active product has not yet been determined, its solubility is radically different from that of enterobactin (Neilands and Expert, unpublished result). Under iron starvation, the *E. chrysanthemi* strain failed to promote the growth of an *E. coli* enterobactin nonproducer carrying the *entE* mutation. Several lines of evidence, however, suggest that DHBA, the intermediate compound in the enterobactin biosynthetic pathway, is also a precursor of the *E. chrysanthemi* siderophore. A spontaneous mutant scored as a multiple aromatic auxotroph from strain 3937 and thus blocked before chorismate did not produce any DHBA-containing compounds. In this mutant, siderophore production and growth under iron starvation could be restored by addition of DHBA. This mutant was also cross-fed with the *E. coli entA* mutant strain, which accumulates the precursor of DHBA. Genetic complementation of the *entA* mutation in *E. coli* could be achieved by cloning the *entA*-like allele from *E. chrysanthemi* 3937 with the plasmid vector pULB113.

Using transposon-induced mutagenesis, we isolated a set of 22 mutants carrying a single insertion of the mini-Mu genome and affected in different steps of the iron assimilation system. In view of the frequency of mutants isolated that displayed the expected phenotype (0.5%), it seems reasonable to assume that the mutations characterized result from insertion of the Mu DNA within the affected genes.

The mutants of classes I and II appeared to be blocked before DHBA in the siderophore biosynthetic pathway, since no catechol could be detected in the supernatant fluid harvested from low-iron cultures. Unlike the multiple aro-

matic auxotroph, these mutants failed to use DHBA as a growth factor and were not cross-fed with the *E. coli entA* strain. These data led us to suppose that the mutations responsible for this phenotype have a polar effect; the genes involved in the biosynthesis of the catechol-type siderophore would be clustered in an operon.

The production of siderophore was found to be associated with overproduction of three outer membrane polypeptides migrating in the 80,000-dalton range. The polypeptide with a molecular weight of 82,000, which appeared to be missing in the outer membrane of the three mutants of class II, which had lost the ability to use the wild-type siderophore, is likely to be the specific envelope receptor for this high-affinity iron transport system. Although the total number of mutants characterized is low, the fact that these three mutants are also impaired in siderophore biosynthesis may be of significance: the gene encoding the putative outer membrane receptor might be transcribed from the same promoter as the genes involved in siderophore biosynthesis. This was actually demonstrated for the ColV plasmid-borne aerobactin genetic system (12), whereas the genes for the biosynthesis of enterobactin and the gene encoding the corresponding outer membrane receptor FepA are transcribed from independent promoters (15).

Three additional classes of mutants were defined. Class III mutants, which failed to use the exogenous siderophore but still produced the 82,000-dalton outer membrane protein, should be altered in internalization or dissociation of the ferric complex. The mutants of class IV, which could cross-feed the class I mutants but not the transport mutants, might be blocked in a late step of siderophore biosynthesis. Those of class V, which were unable to enhance the growth of any siderophore auxotroph, might be unable to excrete any biologically active compound.

Our data suggest that the genes required for the iron system are mostly located in the same region on the chromosome map of *E. chrysanthemi* 3937. All the mutants of classes I and II as well as those of classes III and V (data not shown) could be corrected by receiving a DNA sequence of 35.5 kb contained in the R-prime plasmid R'4.

The study of the behavior of the mutant strains after infection of *Saintpaulia* plants cultured in test tubes provides evidence for a role of microbial iron assimilation in the systemic stages of *E. chrysanthemi* in plant infection. We found a correlation between the decrease in virulence and the loss of any function involved in the catechol-dependent iron transport system. Furthermore, in the two cases that have been studied so far, restoration of the missing iron function partially restored virulence. The fact that only partial virulence was recovered seems not to result from the loss of the cloned iron genes, which appeared to be stable in the bacteria growing in planta. Although no clear explanation for this result can now be provided, it is possible that integration of an R-prime into the chromosome of the transconjugant clones prevents the bacteria from thriving in planta.

All the mutants, unlike the parental strain, failed to induce systemic symptoms, whereas they remained able in some cases to macerate the inoculated leaf. It should be pointed out that they also remained able to macerate isolated organs, such as potato tubers or leaves removed from normal plants. Therefore, it seems that some specific signal emerging from the interaction between the living plant and its pathogen can determine the final outcome of the infection. The delay observed for the appearance of disease symptoms in a certain number of infected plants with mutant strains would support such an interpretation. At this time, any explanation of the data would be speculative. However, we wonder if the loss of virulence results from the inability of the mutant cells to grow efficiently in the leaf tissue because of the lack of free iron, as is probably the case in plant tissue. Mutations altering iron transport might have the same effect as some other auxotrophy mutation, and upon infection with such mutants, the plant could react with some general defense mechanism. Furthermore, it may be possible that *E. chrysanthemi* triggers a specific reaction in the plant which could be responsible for iron deficiency at some particular sites and would make the bacteria unable to compete under such conditions, to go forward within the vessels. Although no high-affinity chelating compound has yet been shown to be synthesized in situ in plants, it may be assumed, particularly in vascular diseases, that iron status in the host may influence the course of infection.

ACKNOWLEDGMENTS

We thank Loïc Malek-Ghassemi and Marc Jullien for the axenically grown *Saintpaulia* plants, Antony Pugsley and all the colleagues of our laboratory for stimulating discussions, and Paul Gill and Joe Neilands for their critical reading of the manuscript.

This work was supported by the Institut National de la Recherche Agronomique and the Ministère de l'Agriculture-DGER (Action incitative Aliment 2000, no. 61410).

LITERATURE CITED

1. **Arnow, L. E.** 1937. Colorimetric determitermination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
2. **Barash, I., et al.** 1987. Effect of iron deficiency on Verticillium wilt diseases and on *in vitro* production of siderophores and organic acid by *Verticillium dahliae*, p. 17–18. *In* 4th International Symposium on Iron Nutrition and Interactions in Plants. University of New Mexico, Albuquerque.
3. **Barth, P. T., N. J. Grinter, and D. E. Bradley.** 1978. Conjugal transfer system of plasmid RP4: analysis by transposon 7 insertion. J. Bacteriol. **133**:43–52.
4. **Bertheau, Y., E. Madjidi-Hervan, A. Kotoujansky, C. Nguyen-The, T. Andro, and A. Coleno.** 1984. Detection of depolymerase isoenzymes after electrophoresis or electrofocusing, or in titration curves. Anal. Biochem. **139**:383–389.
5. **Bilkey, P. C., B. H. McCown, and A. C. Hildebrandt.** 1978. Micropropagation of African violet from petiole cross-sections. HortScience **13**:37–38.
6. **Bullen, J. J., and E. Griffiths (ed.).** 1987. Iron and infection: molecular, physiological and clinical aspects. John Wiley & Sons, New York.
7. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
8. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62**:1159–1166.
9. **Cody, Y. S., and D. C. Gross.** 1987. Outer membrane protein mediating iron uptake via pyoverdin$_{pss}$, the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. J. Bacteriol. **169**:2207–2214.
10. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24**:383–409.
11. **Csáky, T. Z.** 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. **2**:450–454.
12. **de Lorenzo, V., A. Bindereif, B. H. Paw, and J. B. Neilands.** 1986. Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in *Escherichia coli* K-12. J. Bacteriol. **165**:570–578.
13. **Expert, D., and A. Toussaint.** 1985. Bacteriocin-resistant mutants of *Erwinia chrysanthemi*: possible involvement of iron acquisition in phytopathogenicity. J. Bacteriol. **163**:221–227.

14. **Expert, D., E. Schoonejans, and A. Toussaint.** 1986. Cell surface mutants of *Erwinia chrysanthemi*: possible involvement of iron acquisition in phytopathogenicity. NATO Adv. Study Inst. Ser. Ser. A Life Sci. **117**:261–271.
15. **Fleming, T. P., M. Schrodt Nahlik, and M. A. McIntosh.** 1983. Regulation of enterobactin iron transport in *Escherichia coli*: characterization of *ent*::Mu *d*(Ap^r *lac*) operon fusions. J. Bacteriol. **156**:1171–1177.
16. **Hamon, Y., and Y. Peron.** 1961. Les propriétés antagonistes réciproques parmi les *Erwinia*. Discussion de la position taxonomique de ce genre. C. R. Acad. Sci. **253**:913–915.
17. **Howe, M. M.** 1973. Prophage deletion mapping of bacteriophage Mu-1. Virology **54**:93–101.
18. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
19. **Klötz, L., and B. H. Zimm.** 1972. Size of DNA determined by viscoelastic measurements: results on bacteriophages, *Bacillus subtilis* and *Escherichia coli*. J. Mol. Biol. **72**:779–800.
20. **Kotoujansky, A., M. Lemattre, and P. Boistard.** 1982. Utilization of a thermosensitive episome bearing transposon Tn*10* to isolate Hfr donor strains of *Erwinia carotovora* subs. *chrysanthemi*. J. Bacteriol. **150**:122–131.
21. **Kotoujansky, A.** 1987. Molecular genetics of pathogenesis by soft-rot erwinias. Annu. Rev. Phytopathol. **25**:405–430.
22. **Leong, S. A., and J. B. Neilands.** 1982. Siderophore production by phytopathogenic microbial species. Arch. Biochem. Biophys. **218**:351–359.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1**:27–46.
26. **Neilands, J. B., and S. A. Leong.** 1986. Siderophores in relation to plant growth and disease. Annu. Rev. Plant Physiol. **37**: 187–208.
27. **Pugsley, A. P., and P. Reeves.** 1976. Characterization of group B colicin-resistant mutants of *Escherichia coli* K-12: colicin resistance and the role of enterochelin. J. Bacteriol. **127**:218–228.
28. **Résibois, A., M. Colet, M. Faelen, E. Schoonejans, and A. Toussaint.** 1984. PhiEC-2, a new generalized transducing phage of *Erwinia chrysanthemi*. Virology **137**:102–112.
29. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7**:445–456.
30. **Schoonejans, E., D. Expert, and A. Toussaint.** 1987. Characterization and virulence properties of *Erwinia chrysanthemi* lipopolysaccharide-defective, PhiEC2-resistant mutants. J. Bacteriol. **169**:4011–4017.
31. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology **1**:784–790.
32. **Slade, M. B., and A. I. Tiffin.** 1984. Biochemical and serological characterization of *Erwinia*. Methods Microbiol. **15**:227–293.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
34. **Thiry-Braipson, J., C. d'Agostino, and L. Ledoux.** 1979. Characterization of a bacteriocin produced by *Erwinia uredovora*. Arch. Int. Physiol. Biochim. **87**:845–846.
35. **Van Gijsegem, F., and A. Toussaint.** 1982. Chromosome transfer and R-prime formation by an RP4::mini-Mu derivative in *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Plasmid **7**:30–44.
36. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection disease. Physiol. Rev. **64**:65–102.
37. **Winkelmann, G., D. Van der Helm, and J. B. Neilands (ed.).** 1987. Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft mbH, Weinheim, Federal Republic of Germany.
38. **Young, I. G., L. Langman, R. K. J. Luke, and F. Gibson.** 1971. Biosynthesis of the iron transport compound enterochelin: mutants of *Escherichia coli* unable to synthesize 2,3-dihydroxybenzoate. J. Bacteriol. **106**:51–57.