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A Locus Determining Pathogenicity of *Xanthomonas campestris* Is Involved in Lipopolysaccharide Biosynthesis

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A pathogenicity locus in *Xanthomonas campestris* pv. *campestris* has been shown to comprise two genes which mediate biosynthesis of the bacterial lipopolysaccharide (LPS) but not extracellular polysaccharide. Mutants with Tn*5* insertions in either gene showed alterations in the electrophoretic patterns of both water-soluble and phenol-soluble LPS forms, which suggested defects in the biosynthesis of the core oligosaccharide component. On gel chromatography, core oligosaccharides of the mutants were of apparently lower molecular weight than those from the wild type. Furthermore, the content of mannose and glucose, sugars characteristic of the core oligosaccharide, were significantly lower in the water-soluble LPS of the mutants. Because of their role in LPS core biosynthesis, the two genes were called *rfaX* and *rfaY*. *rfaX* mutants show altered behavior in a range of host and non-host plants such that the number of recoverable bacteria drop within the first 24 h after inoculation. In contrast, the behavior of *rfaY* mutants only differed from the wild type in *Datura*, a nonhost plant in which the growth of the wild type is severely attenuated. The predicted protein RfaY showed significant sequence homology to a sub-family of RNA polymerase σ factors which are involved in extracytoplasmic functions.

Additional keywords: alternate sigma factor, core oligosaccharide biosynthesis.

Surface and extracellular polysaccharides of phytopathogenic bacteria have been shown to have a role in pathogenesis in a number of different diseases. Mutants either defective in both lipopolysaccharide (LPS) and exopolysaccharide (EPS) synthesis or in LPS synthesis alone and with reduced virulence have been isolated from all the major genera of Gram-negative pathogens (Drigues et al. 1985; Schoonejans et al. 1987; Kao et al. 1990; Kingsley et al. 1993). LPS and EPS are thought to contribute to bacterial growth and survival in planta by acting as a barrier to plant defenses, by creating a favorable environment for bacterial growth and possibly by modulating host reactions (Kingsley et al. 1993; Dazzo 1991; Niehaus et al. 1993). *Xanthomonas campestris* pv. *campestris,* the causal agent of black rot of crucifers (Williams 1980), produces the exopolysaccharide xanthan; the biochemistry and genetics of xanthan biosynthesis have been the subject of considerable study. Comparatively little is known, however, about LPS structure and biosynthesis in *Xanthomonas*.

Two types of LPS have been reported in *Xanthomonas*, a phenol-soluble LPS and a water-soluble LPS. The phenol-soluble LPS contains D-rhamnose and 3-acetamido-3,6-dideoxygalactose as major components (Hickman and Ashwell 1966). The water-soluble LPS contains glucose, mannose, galactose, rhamnose, and galacturonic acid along with 2-keto-3-deoxyoctulosonic acid but no heptose (which is found in the LPS of enteric bacteria) (Volk 1966; Köplin et al. 1993). Genes involved in the biosynthesis of both xanthan and LPS have been isolated and characterized. These genes include those mediating the biosynthesis of UDP-glucose and GDP-mannose (which are common precursors for LPS and xanthan) (Köplin et al. 1992). Another of these, *opsX* (Kingsley et al. 1993) from *X. campestris* pv. *citrumelo* has a predicted protein product which shows amino acid sequence homology to both Lsi-1 of *Neisseria gonorrhoeae* and RfaQ of *Escherichia coli*, which are involved in LPS core biosynthesis. Mutation of *opsX* causes abolishes virulence in citrus but not bean, associated with rapid killing of all the bacteria. A 3.9-kb DNA region of *X. campestris* pv. *campestris* encoding a set of enzymes involved in the synthesis of dTDP-rhamnose, the precursor for the rhamnose moieties of the O-antigen component of LPS, has also been described (Köplin et al. 1993). Mutations in this region cause the production of rough LPS (lacking O-antigen) and lower the production of xanthan without changing its composition. The effects of these mutations on pathogenicity were not reported.

In a previous publication we described a locus in *X. c.* pv. *campestris,* defined by transposon mutagenesis, which is involved in pathogenicity to turnip seedlings (Osbourn et al. 1990). Mutations at the locus reduce or abolish symptom production in a range of pathogenicity tests using seeds, seedlings, or mature plants (Osbourn et al. 1990; Newman et

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The nucleotide sequence of *rfaX* was originally deposited in the EMBL database under accession number M55152 and delineator Xcpathx. The sequence of *rfaY* was deposited in the EMBL/GenBank/DDBJ databases under accession number U19896.

al. 1994; M. J. Daniels and C. E. Barber, unpublished). The function of the genes within the locus was unknown; the levels of extracellular enzymes and xanthan, factors implicated in virulence (reviewed by Dow and Daniels 1994) were not affected. In this paper we report that the locus comprises two genes which are both involved in the biosynthesis of the core component of LPS. The two genes were called *rfaX* and *rfaY*. The predicted protein product of *rfaX*, whose sequence we reported previously (Osbourn et al. 1990), has no amino acid sequence homology to proteins in the databases. The predicted protein product of *rfaY*, however, shows amino acid sequence homology to a subfamily of RNA polymerase σ factors suggested to regulate extracytoplasmic functions (Lonetto et al. 1994). The behavior of *rfaX* and *rfaY* mutant strains in the mature leaves of host and nonhost plants suggests that LPS performs an important barrier function against plant antibacterial agents in the early stages of infection.

RESULTS

Behavior of mutants in mature plants.

The wild-type strain 8004 and mutant strains 8530 (*rfaX*::Tn*5*), 8541 (*rfaY*::Tn*5*), and 8540 (a control strain which carries a Tn*5* insertion outside the locus) are described in Table 1. These strains were introduced into the mature leaves of the host plants turnip (*Brassica campestris*) cv. Just Right and *Arabidopsis thaliana* ecotype Columbia (Parker et al. 1993) and the nonhosts *Datura stramonium*, pepper (*Capsicum annuum*) line ECW-10R, and tomato (*Lycopersicon esculentum*) cv. Moneymaker at 10^5 or 10^6 CFU per milliliter. In all plants, the growth of strain 8540 was indistinguishable from the wild-type strain 8004; bacterial numbers increased over the first 24 h after inoculation albeit to different extents in host and nonhost plants (Fig. 1). In contrast, the numbers of viable bacteria of strain 8530 dropped by an order

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
8004	Rif-14, wild type	Daniels et al. 1984
8539, 8540, C4	8004::Tn*5* mutants derived by marker exchange of Tn*5* insertions 8,17, and C4, respectively, in pIJ3122, pathogenic to seedlings	Osbourn et al. 1990; unpublished
8530	8004::Tn*5* mutant derived by marker exchange of Tn*5* insertion 29 in pIJ3122, clearly reduced pathogenicity in seedlings, *rfaX*	Osbourn et al. 1990
8541	8004::Tn*5* mutant derived by marker exchange of Tn*5* insertion 34 in pIJ3122, partially reduced pathogenicity in seedlings, *rfaY*	Osbourn et al. 1990
pIJ3122	*X. c.* pv.*campestris* DNA carrying pathogenicity locus, cloned in pLAFR3	Osbourn et al. 1990
pLGUS	pLAFR3 derivative carrying the *uidA* gene driven by the *lac* promoter	S. Soby and M. J. Daniels, unpublished

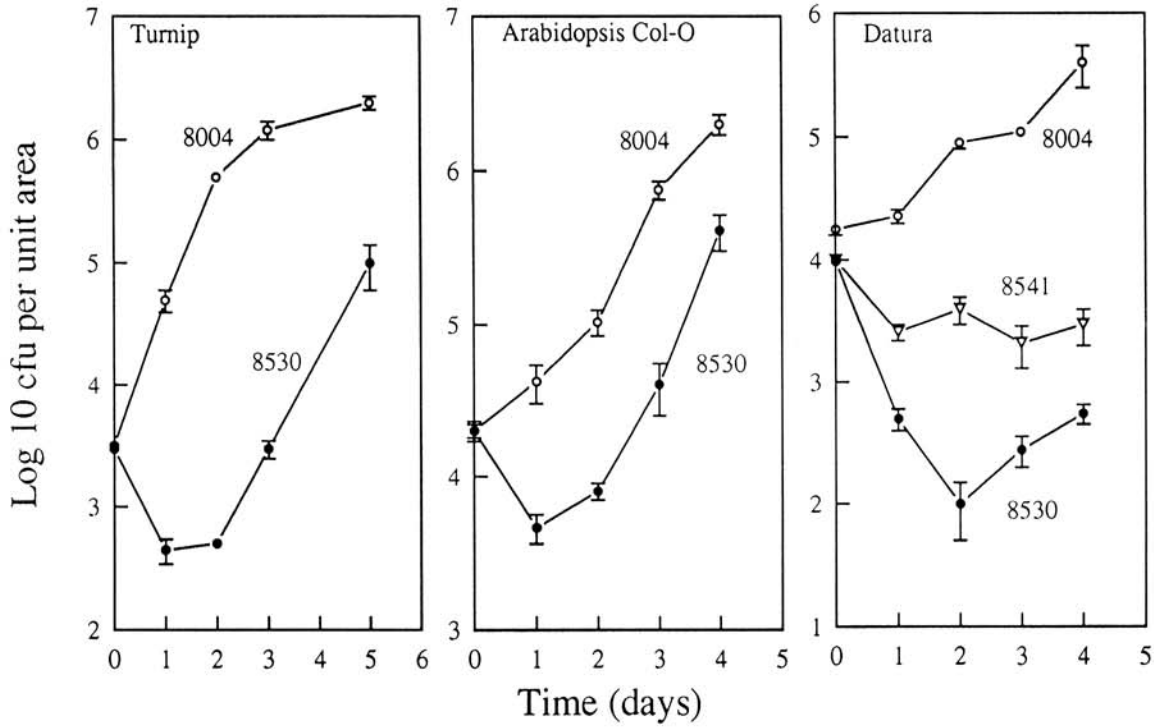

Fig. 1. Growth of strains of *Xanthomonas campestris* pv. *campestris* in the mature leaves of the host plants turnip (*Brassica campestris* cv. Just Right) and *Arabidopsis thaliana* ecotype Columbia (Col-O) and the nonhost *Datura stramonium*. The strains are 8004 (wild type), 8530 (*rfaX*), and 8541 (*rfaY*). Leaves were infiltrated with suspensions of bacteria at 10^5 (turnip) or 10^6 CFU/ml. The mean and standard deviation of at least four separate measurements at each time point are given.

of magnitude after inoculation into all plants. Between 24 and 48 h, bacterial numbers began to increase in turnip and *Arabidopsis*, although in *Datura*, pepper and tomato bacterial numbers continued to decline. The growth of strain 8541 was indistinguishable from strains 8540 and 8004 in all plants except *Datura*, where growth characterisitics were intermediate between those of 8004 and 8530. Strain 8541 shows only partial reduction in pathogenicity to turnip seedlings (Osbourn et al. 1990). In plate counts of bacteria recovered from plants, it was evident that colonies of 8530 were smaller than those of 8004, particularly when recovered from nonhost plants. This difference was not seen in dilutions of cells grown in nutrient broth.

The differential behavior in plants of mutants and wild type was further analyzed by the use of strains 8004/pLGUS and 8530/pLGUS (Table 1), which constitutively express β-glucuronidase (GUS) activity. These strains were inoculated into turnip and after 24 h the leaves were homogenized as for the viable count measurements. All of the GUS activity was extracted from the plant debris by homogenization and the level of GUS activity was approximately fivefold higher per unit area of leaf in leaves inoculated with 8004/pLGUS than with 8530/pLGUS. This suggests that the viable count measurements are not affected by differential binding of the mutants to plant debris and that true differences in bacterial numbers occur.

The behavior of strain 8530 in turnip or *Arabidopsis* was not altered by coinoculation or preinoculation with the wild type, by inoculation in 50 mM PIPES buffer at pH 7 or by inoculation in solutions containing catalase (50 μg/ml) or superoxide dismutase (100 units/ml). These results suggest that low pH conditions or active oxygen species likely to occur in plant intercellular spaces are not responsible for the loss of viability. In addition they indicate that wild-type bacteria cannot suppress the plant response which causes the loss in viability and are not able to complement the defect in the mutant bacteria by provision of an extracellular component (Kamoun and Kado 1990). Strain 8530 bacteria recovered from plants at 72 h after inoculation were all kanamycin resistant (showing no loss of the Tn*5* transposon) and when reinoculated into plants again showed the decrease in bacterial numbers. This demonstrates that the recovery of the ability to grow in hosts is not due to the selection of variants. At an inoculum level of 10^7 or 10^8 CFU/ml all strains elicited a hypersensitive response in pepper and tomato within 24 h of inoculation. This indicates that the locus does not contain *hrp* genes which are involved in both hypersensitive response and pathogenicity in many phytopathogens including *Xanthomonas* (Arlat et al. 1991).

Table 2. Sensitivity of wild type *Xanthomonas campestris* strain 8004 and mutant strain 8530 (*rfaX*) to antimicrobial agents

	Minimum inhibitory concentration (μM)[a]		
Strain	**Plumbagin**	**Novobiocin**	**FCCP**
8004	>250	4.8-8.0	1.2
8530	50	0.8-1.6	0.3

[a] Minimum inhibitory concentrations were determined by assessment of the growth of bacteria in 10 ml of peptone-yeast extract-glycerol broth at 28°C at a range of inhibitor concentrations.

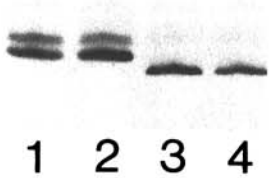

Fig. 2. Lipopolysaccharide (LPS) profiles of strains of *Xanthomonas campestris* pv. *campestris* as revealed by SDS-PAGE and silver-periodate staining of nuclease and proteinase K-treated whole cells. Only the lower part of the gel is shown. The lanes are 1: strain 8004 (wild type); 2: strain 8540 (control Tn*5* insertion); 3: strain 8530 (*rfaX*); 4: strain 8541 (*rfaY*).

Biochemical characterization of mutants.

Initial biochemical comparisons were done using the wild-type strain 8004 and strain 8530, as these strains showed the most marked differences in behavior in plants. No differences between the strains were seen in sensitivity to low pH estimated by plating out dilutions on nutrient media plates adjusted to different pH values with HCl or by measuring survivability in buffers at a range of pH values from 5 to 7. Furthermore, mutant and wild type showed no differences in membrane proton permeability and proton efflux capacity. Membrane potential was estimated by use of a fluorescent cyanine dye to be −110 mV in both strains. The mutant did not differ from the wild type in its sensitivity to the detergents Tween 20 and Nonidet NP40 and both strains were insensitive to the redox-cycling agent paraquat at concentrations up to 500 μM and to hydrogen peroxide (7 mM, 15 min). However the mutant was more sensitive to the action of the antibiotic novobiocin, the uncoupler carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) and the redox-cycling agent plumbagin. Minimum inhibitory concentration values are given in Table 2. In N broth addition of 25 μM plumbagin (a sublethal level) to growing cultures increased the doubling time from 1.9 to 2.8 (8004) or 4.8 h (8530). In contrast strain 8530 was not sensitive to Triton X-100 although the wild type was sensitive. Plating efficiencies of the wild type on NYGA containing 0.5% Triton X-100 were only 25% of that on NYGA alone, whereas no differences in plating efficiency were seen with strain 8530. In all of these tests the more sensitive strain showed smaller and more variable colony sizes. The sensitivity of strain 8541 to plumbagin, novobiocin, and FCCP was intermediate between that of the wild-type strain 8004 and strain 8530.

The changes in susceptibility to a variety of agents with different modes of antimicrobial action suggested an impairment in the barrier function of the bacterial cell which would normally exclude such compounds. This barrier function involves the cell envelope and principally the LPS. (Nikaido and Vaara 1985). Envelope preparations were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining, but no differences between the protein profiles of strains 8004, 8530, and 8541 were evident. However, when LPS profiles of the cells were analyzed by SDS-PAGE, major differences in the banding pattern after silver-periodate staining were seen in the lower part of the gel (Fig. 2). Two bands were seen in the wild type and strain 8540, whereas strains 8530 and 8541 had a single band of increased mobility. This strongly suggested that the pathogenicity locus was involved in LPS biosynthesis.

Extraction and fractionation of LPS from wild type and mutant bacteria.

LPS was extracted from the bacteria by the hot phenol method and the aqueous and phenol phases dialyzed separately until all traces of phenol had disappeared. Preliminary fractionation of the water-phase LPS was as described by Barton-Willis et al. (1984). Nucleic acid in the aqueous phase was removed by precipitation with hexadecyltrimethlammonium bromide (CTAB), and the LPS was precipitated from the supernatant with ethanol. Further fractionation was by Sepharose 6B chromatography in triethylamine buffer (Carlson et al. 1978). The elution was monitored by the phenol-sulphuric acid assay for carbohydrate (Fig. 3A) and for LPS by SDS-PAGE and silver-periodate staining. In all strains, LPS and carbohydrate coeluted from the column as a single broad peak in the same (included) volume. There were sometimes other carbohydrate-containing materials which eluted in the low molecular weight exclusion volume of the column. However, these did not contain LPS as judged by SDS-PAGE and silver-periodate staining and were not studied further. On SDS-PAGE, purified LPS showed the same patterns as in the whole cell extracts (Fig. 4A); two bands of high mobility were seen in the wild type and strain 8540, whereas 8530 and 8541 both had a single band of increased mobility. Comparison of these patterns with those previously published for *Xanthomonas* (Kingsley et al. 1993; Köplin et al. 1993; Ojanen et al. 1993) suggests that (i) the wild-type LPS comprises core oligosaccharides attached to the lipid A moiety but with no O-antigen (which would give a lower mobility on the gel) and (ii) the mutant is defective in the synthesis of the core component. The LPS containing fractions were pooled, dialyzed against water and lyophilized to yield a fluffy white powder. The recovery of LPS was lower from strains 8530 and 8541 than from the wild type or strain 8540.

After an insoluble component was removed by centrifugation, the dialyzed phenol-soluble fraction of the bacterial extract was concentrated by lyophilization. On SDS-PAGE, this crude fraction of the wild type had components of both high and low mobility as detected by silver staining (Fig. 4B). Treatment with pronase eliminated some minor bands, but the major bands were unaffected, suggesting that they were probably LPS. Pronase-treated fractions from 8541 and 8530 showed only a single band of high mobility. On Sepharose 6B chromatography of this fraction from all strains, the carbohydrate elution profile (Fig. 3B) matched that of the LPS elution determined by SDS-PAGE . LPS of the wild type eluted as a broad peak just after the elution volume for blue dextran, whereas LPS of the mutants 8530 and 8541 eluted in a larger volume. The LPS profiles of the peak fractions of the wild type and mutants (Fig. 4B) were identical to those of the appropriate pronase-treated crude phenol phases. The mobility on gels and elution profile from the Sepharose 6B column suggests that the phenol-soluble LPS from the wild type does contain an O-antigen (in contrast to the water-phase LPS) and that in the LPS from the mutants, defects in the core oligosaccharide lead to the absence of this component. As the recovery of phenol-phase LPS was, however, much lower than that of the water-phase LPS, this material was not studied further.

Analysis of the water-phase LPS from mutant strain 8530 and wild type.

Mild acid hydrolysis cleaves the ketosidic bond between 3-deoxy-D-*manno*-2-octulosonic acid (KDO) residues and be-

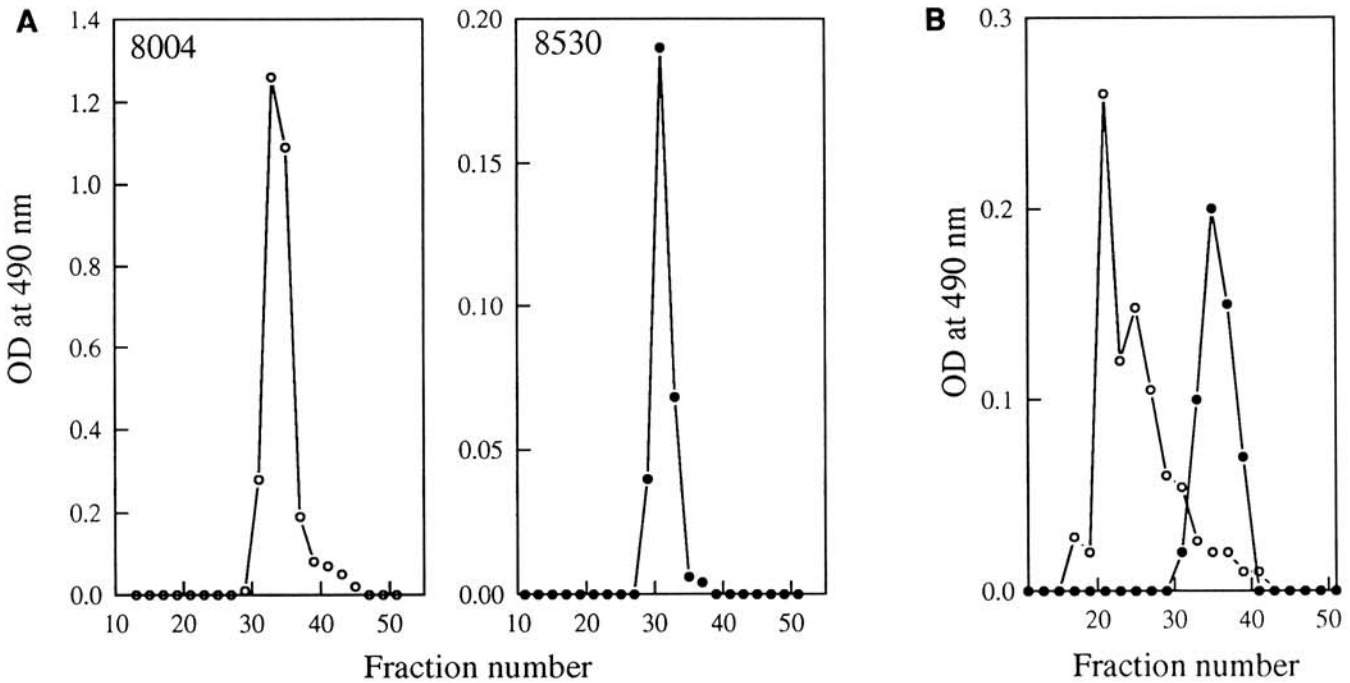

Fig. 3. Sepharose 6B gel filtration of lipopolysaccharide (LPS) preparations from *Xanthomonas campestris*. The elution profile was determined by assay of the fractions for carbohydrate by the phenol-sulphuric acid method. Blue dextran eluted in fraction 16 and arabinose (low molecular weight marker) in fraction 45. **A,** LPS from the water phase of the hot phenol-water extract after CTAB treatment and ethanol precipitation. **B,** LPS from the phenol phase of the hot phenol-water extract. Open symbols: wild type strain 8004; closed symbols: strain 8530 (*rfaX*).

tween KDO and lipid A in the LPS of enteric bacteria to release soluble core oligosaccharides and core plus O-antigen. When the *Xanthomonas* water-phase LPS samples were subjected to mild acid hydrolysis (1% acetic acid, 100°C, 3 h) a precipitate, believed to be the lipid A, formed in both samples. After this was removed by centrifugation, the supernatant solution was subjected to size exclusion chromatography on Sephadex G-50. All of the carbohydrate eluted late in the included volume of the column but before the elution position of monosaccharides (Fig. 5). This behavior on Sephadex G-50 is typical of core oligosaccharides (Barton-Willis et al. 1984) and again suggests an absence of O-antigen which would probably elute in the blue dextran void volume in this column (Bukharov et al. 1993). The oligosaccharides derived from the LPS of mutant 8530 were of apparently lower molecular weight than those of the wild type. This is the expected result if 8530 were defective in core oligosaccharide completion.

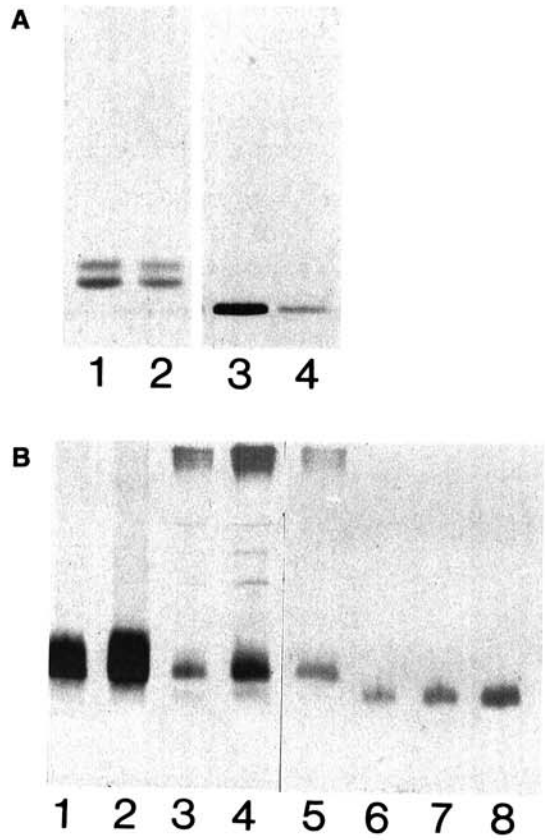

Fig. 4. Analysis of purified LPS preparations by SDS-PAGE and silver-periodate staining. **A,** LPS from the water phase of the hot phenol-water extract after Sepharose 6B chromatography. The lanes are 1: strain 8004 (wild type); 2: strain 8540 (control Tn*5* insertion); 3: strain 8530 (*rfaX*); 4: strain 8541 (*rfaY*). **B,** LPS preparations from the phenol phase of the hot phenol-water extract. The lanes 1 and 2 are markers of purified water phase LPS from strains 8004 and 8540 (control Tn*5* insertion) at high loading (showing absence of bands of low mobility); lanes 3 and 4: crude phenol phase LPS from strains 8004 and 8540; 5: Sepharose 6B purified phenol phase LPS from strain 8004; 6 and 7: Sepharose 6B purified phenol phase LPS from strain 8530 (*rfaX*) at two different loadings; 8: Sepharose 6B purified phenol phase LPS from strain 8541 (*rfaY*).

Monosaccharides were released from 1 mg of purified lyophilized LPS samples by hydrolysis with 2 M trifluoroacetic acid and hydrolysates prepared for TLC analysis as outlined in experimental procedures. TLC on silica plates in solvent A followed by staining with *N*-(1-naphthyl)ethylenediamine dihydrochloride revealed the presence of approximately equal amounts of hexosamine in both samples but a reduced level of hexose in strain 8530 LPS. Both samples lacked rhamnose, galacturonic acid, and glucuronic acid showing that the samples were not contaminated with xanthan (which contains D-glucuronic acid) and that both LPS fractions lacked the O-antigen (which contains L-rhamnose and D-galacturonic acid [Bukharov et al. 1993]). Minor spots of R_f 0.24 and 0.91 were of equal intensity. TLC on cellulose plates followed by staining with alkaline silver nitrate showed that the major hexoses in the wild type are glucose and mannose with no detectable galactose. In the mutant strain 8530, there were reduced levels of both of these sugars, although approximately the same level of a hexosamine with the same mobility as D-glucosamine. These analyses are consistent with those of Köplin et al. (1993) who demonstrated that a rough mutant of *X. campestris* pv. *campestris* with the same LPS profile as the water-phase LPS from strain 8004, had glucosamine, glucose, and mannose as major sugars. No sugars with the same mobility as heptoses were detected. The properties of the water-phase LPS from strain 8541 were essentially the same as those from strain 8530.

Sequence analysis of a further gene in the pathogenicity locus.

Since the pathogenicity locus mediates LPS core assembly but not extracellular polysaccharide (xanthan) biosynthesis we have termed it the *rfa* locus in accordance with the established nomenclature for LPS biosynthetic genes in enteric

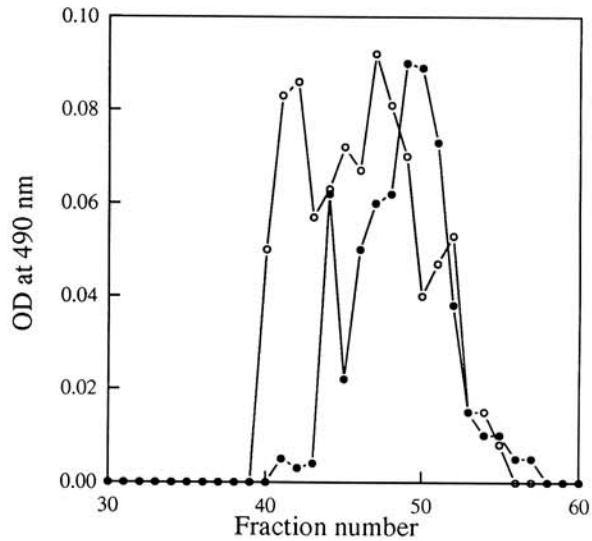

Fig. 5. Sephadex G-50 gel filtration of core oligosaccharides released from purified water-phase LPS by mild acid treatment. The elution was monitored with the phenol-sulphuric acid assay. Closed symbols: strain 8530 (*rfaX*); open symbols: strain 8004 (wild type). Blue dextran and the monosaccharide arabinose eluted with maximum concentration in fractions 20 and 55, respectively.

bacteria. The locus is bounded by Tn*5* insertions 8 and C4 which both give wild-type pathogenicity and LPS profiles and are approximately 2.1-kb apart (Fig. 6). Tn*5* insertion 29 is within the gene whose sequence has already been reported (Osbourn et al. 1990) which we have called *rfaX*. The nucleotide sequence of the rest of the locus was determined and predicted an open reading frame (ORF) of 651 nucleotides in the same orientation as *rfaX*. Analysis of the sequence using the Frame program (Bibb et al. 1984) was consistent with the presence in this region of an ORF in this orientation. The ORF spans both *Pst*I sites and the site of Tn*5* insertion 34; the corresponding gene was called *rfaY* (Fig. 6). The predicted primary translation product of this gene is a protein of 217 amino acids and M_r 24,196. An alternative translational initiation site (a methionine residue at position 19 in the longer sequence) would give a protein of 199 amino acids and M_r 22,173. A survey of the databases using the BLAST algorithm (Altschul et al. 1990) revealed homology of RfaY with AlgU, a putative alternate sigma factor of *Pseudomonas aeruginosa* controlling expression of *algD* (Martin et al. 1993) and CarQ, a positive regulator of carotenoid biosynthesis in *Myxococcus xanthus* (McGowan et al. 1993). RfaY had 52% similarity and 26% identity to AlgU and 39% similarity and 28% identity to CarQ, P values were 6.8×10^{-13} and 1.1×10^{-10}, respectively. Sequence similarity of AlgU and CarQ to the defined sigma factors SigE of *Streptomyces coelicolor* and σ^E of *Escherichia coli* has suggested that they are both sigma factors belonging to a subfamily involved in the regulation of extracytoplasmic functions (Lonetto et al. 1994). RfaY has amino acid sequence homology throughout its length to all members of this family. Sequence alignments with some of the members are shown in Figure 7. Using the MOTIFS program, a potential helix-turn-helix motif was detected in RfaY involving residues 163 to 187.

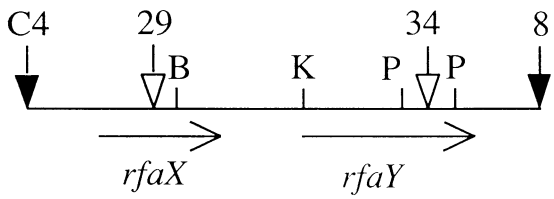

Fig. 6. Physical map of the pathogenicity locus showing the restriction sites for *Bam*HI (B), *Kpn*I (K), and *Pst*I (P) and the positions of the different transposon insertions (triangles). Transposon insertions giving a wild-type LPS profile and pathogenicity are represented by closed triangles, those giving altered LPS profiles and altered pathogenicity by open triangles. The positions of the predicted open reading frames for *rfaX* and *rfaY* are also shown. The scale bar represents 1 kb.

Homologues of *rfaX* and *rfaY* in other Gram-negative bacteria.

Probes carrying the full coding sequences of *rfaX* and *rfaY* were prepared by PCR amplification from the clone pIJ3122 and were used in in Southern blotting of *Eco*RI-digested

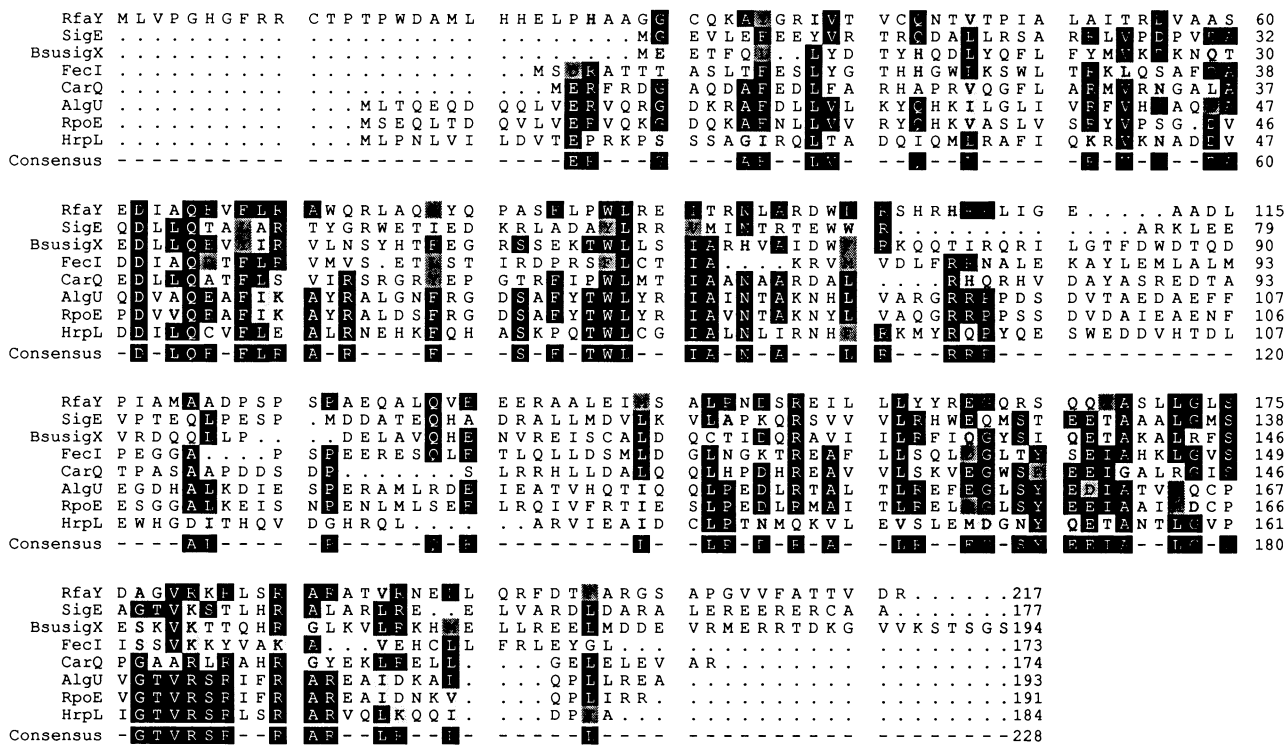

Fig. 7. Similarities between RfaY and members of a subfamily of RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. Sequences were aligned using the algorithm PILEUP (Devereux et al. 1984). Residues found in the majority of the sequences shown are highlighted in black and represented in the consensus sequence. Conservative substitutions are indicated by the shaded boxes. Abbreviations: Sige; SigE of *Streptomyces coelicolor* (Lonetto et al. 1994); Bsusigx: SigX of *Bacillus subtilis* ORF X20 of Sorokin et al. (1993); Feci: FecI of *E. coli* (van Hove et al. 1990); Carq: CarQ of *Myxococcus xanthus* (McGowan et al. 1993); Algu: AlgU of *Pseudomonas solanacearum* (Martin et al. 1993); Rpoe: RpoE of *E. coli* (cited in Lonetto et al. 1994); Hrpl: HrpL of *Pseudomonas syringae* pv. *syringae* (Xiao et al. 1994). Accession numbers for the sequences are given in Lonetto et al. (1994).

chromosomal DNA of *X. campestris* pathovars and other bacteria as described in experimental procedures. The DNA of all strains of *X. campestris* pv. *campestris* showed strong hybridization to the *rfaX* probe as did *X. campestris* pv. *armoraciae*. A single hybridizing band was seen. Weak hybridization was seen with *X. campestris* pvs. *vitians, vesicatoria, malvacearum,* and *holcicola*. At the stringency used no hybridization was evident with *Pseudomonas syringae* pv. *coronafaciens* and *maculicola*, *Escherichia coli*, *Erwinia carotovora, Erwinia salicis,* or *Erwinia chrysanthemi.* Essentially the same results were obtained with the *rfaY* probe.

DISCUSSION

Our data strongly suggest that the pathogenicity locus which we had previously described mediates the biosynthesis of the core component of LPS and that intact LPS is required for full virulence on a range of plants. The locus comprises two genes; the previously characterized gene (Osbourn et al. 1990) which we have called *rfaX* and *rfaY*, whose characterization we report here. The predicted protein product of *rfaX* shares no homology with other genes in the database and its role in LPS biosynthesis remains obscure. However, the predicted protein product of *rfaY* shows sequence similarity to a family of alternate σ factors which are implicated in controlling extracytoplasmic functions in a number of bacteria (Lonetto et al. 1994). This sequence similarity suggests that RfaY directs the transcription of at least a subset of the *rfa* genes (involved in LPS core biosynthesis) in *X. campestris*. We cannot exclude the possibility, however, that RfaY is involved in transcription of other genes which indirectly affect LPS assembly (see below). At the moment we have no biochemical evidence for this proposed function of RfaY.

Currently nothing is known of the *rfa* genes in *Xanthomonas* spp. or of their organization. In enteric bacteria, the majority of *rfa* genes are organized in a large cluster (reviewed by Schnaitman and Klena 1993). *rfaX* and *rfaY* do not appear to form part of a large *rfa* operon in *X. campestris* since transposon insertions distal to C4 and 8 (Fig. 6) have no apparent effect on LPS structure (and pathogenicity). However in enteric bacteria there are also genes located outside the *rfa* cluster which exert effects on core structure. An example (which closely parallels *rfaY*) is the *rfaH* gene of *E. coli*. This gene encodes a transcriptional activator of both an operon within the *rfa* gene cluster and genes involved in haemolysin synthesis and export and F-factor expression (Bailey et al. 1992). These genes have been considered to comprise a 'virulence regulon.' It will be of interest to determine if *rfaY* is similarly involved in transcription of further genes contributing to pathogenicity in *X. campestris*.

LPS of *rfa* mutants has markedly reduced levels of mannosyl and glucosyl residues. This is consistent with observations that mutation of *xanB*, a gene mediating the biosynthesis of UDP glucose and GDP mannose in *X. campestris*, also gives rise to LPS a modified structure (Köplin et al. 1992). The simplest interpretation of these results is that mannosyl and glucosyl residues are components of the outer core in the two major lipid A-core components of the wild type and their absence leads to a smaller lipid A-core oligosaccharide with increased gel mobility in the mutants. However, as nothing is known of the structure or complexity of the core oligosaccharide(s) in *Xanthomonas*, this inferred biosynthetic relationship remains speculative.

The decrease in viability of the *rfaX* mutant (strain 8530) after inoculation into plants may be related to the increased sensitivity of this strain to inhibitors such as the plant-derived plumbagin. It is not apparently due to increased sensitivity to low pH conditions or to active oxygen species. Many plants may contain preformed compounds which are potentially toxic to bacteria but which are normally excluded from the bacterial cell by a complete LPS structure. Furthermore such compounds may serve to restrict the host range of bacteria as suggested by Kingsley et al. (1993). Mutation of *rfaY*, which gives rise to an intermediate sensitivity to the inhibitors in vitro, affects bacterial behavior only in the nonhost *Datura,* where growth of the wild type is severely restricted, and in the turnip seedling assay (Osbourn et al.1990). The different effects of mutation of *rfaY* and *rfaX* on bacterial viability in planta and sensitivity to inhibitors in vitro are not reflected in gross differences in the LPS forms produced by the mutant strains. However, subtle differences in structure could clearly be present. In addition, the two mutations may have pleiotropic effects which contribute differently to the increased sensitivity to inhibitors and behavior in plants.

The other striking feature of the growth and survival kinetics of strain 8530 in planta is the 'recovery' of the mutant bacteria so that they begin to grow after 24 to 48 h in host plants. The mechanism is obscure but could involve turnover of toxic plant components, the induction of systems for the efflux of such compounds (Ma et al. 1994), and/or other adaptive changes to stress in the bacteria. The fact that preinoculation with the wild type or coinoculation of this mutant with the wild type does not significantly change the growth and survival kinetics of the mutant argues that turnover of toxic plant components may not be a feature of normal pathogenesis. Mechanisms to deal with such compounds may only be induced when they are not effectively excluded from the bacterial cell (when the LPS barrier is defective) (Ma et al. 1994).

The proposed role for RfaY in regulating the biosynthesis of LPS would be completely consistent with its membership (as revealed by amino acid sequence homology) in a subfamily of sigma factors suggested to transcribe genes involved in extracytoplasmic functions (the ECF subfamily, Lonetto et al. 1994). Another member of the subfamily is σ^E from *E. coli*, which is involved in the heat-shock response (Raina et al. 1995; Rouvière et al. 1995). Interestingly, recent work suggests that some members of the σ^E regulon may also be involved in maintaining the integrity of the outer membrane or of the cell wall under normal growth conditions (Rouvière et al.1995). A second common theme amongst the ECF subfamily is that they respond to extracytoplasmic conditions. For example in the plant pathogen *Pseudomonas syringae*, another member of the family (HrpL), which controls transcription of a virulence factor and of the machinery for its extracellular secretion, is inducible by plant extracts (Xiao et al. 1994). Modulation of the activity of RfaY in response to an extracytoplasmic plant-derived signal may lead to modifications of LPS structure (and perhaps other cell surface changes) promoting bacterial survival in planta. Although such changes in LPS have yet to be documented for plant pathogenic bacteria, they clearly occur in symbiotic *Rhizo-*

bium spp. during nodule formation (Kannenberg et al. 1994) and in bacterial pathogens of animals in response to environmental changes (Skurnik and Toivanen, 1993). Other components may be necessary to transduce such an extracytoplasmic signal to the transcriptional apparatus. A candidate transducer in *Xanthomonas* would be RfaX, which contains one potential membrane spanning domain (Osbourn et al. 1990) but clearly many other roles of RfaX are possible.

The proposed function of RfaY (as a σ factor) will be examined in future biochemical work. If RfaY is indeed a σ factor, identification of the genes which depend on it for their transcription will be a valuable step towards a further understanding of the roles of both RfaX and RfaY in pathogenesis.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions.

Strains and plasmids used in this work are listed in Table 1. Bacteria were grown in peptone-yeast extract-glycerol broth (NYGB, Daniels et al. 1984) at 28°C. Infiltration of bacterial suspensions in water into mature leaves of plants was done as described by Collinge et al. (1987). Bacterial numbers in plants were measured as described by Parker et al. (1993). Growth conditions for *Arabidopsis thaliana* Ecotype Columbia were those used by Parker et al. (1993) and for all other plants those used by Collinge et al. (1987) for turnip cultivar Just Right.

Biochemical characterization of mutants.

Proton permeability and proton efflux measurements were made as described by Chen et al. (1993). Membrane potential was estimated by fluorescence quenching of the cyanine dye diS-C_3-(5) as described by Waggoner (1978) using 2.5×10^{-7} M dye and 4×10^7 cells in 2 ml of 0.1 M MOPS/Tris buffer pH 7. Minimum inhibitory concentrations of plumbagin, novobiocin, and FCCP were determined in 10-ml cultures of NYGB in universal bottles. Bacterial envelopes were prepared by centrifugation after lysis of the bacteria in the French pressure cell as described previously (Dow et al. 1987). For determination of LPS profiles of cells, bacteria were lysed and treated with RNase, DNase, and proteinase K as described by Kingsley et al. (1993). Extracts were subjected to SDS-polyacrylamide gel electrophoresis in 15% gels which were stained by the silver-periodate method (Wood et al. 1989).

Extraction, fractionation, and characterization of LPS.

Cells were grown in NYGB medium to an OD_{600} of 0.8 to 1.0, harvested by centrifugation and washed twice with 0.7% sodium chloride and once with water. LPS was extracted by the hot phenol method of Westphal and Jann (1965). The aqueous and phenol phases were dialyzed separately to remove all traces of phenol. Nucleic acid in the aqueous phase was removed by precipitation with hexadecyltrimethylammonium bromide and the LPS was precipitated with ethanol as described by Barton-Willis et al. (1984). The precipitate was recovered by centrifugation and dissolved in 30 mM triethylamine hydrochloride-10 mM EDTA pH 7. Further fractionation was on a Sepharose 6B column (1.5 × 42 cm) equilibrated with 30 mM triethylamine hydrochloride pH 7. Fractions of 1.4 ml were collected at a flow rate of 6 ml per h. Fractions were assayed for carbohydrate by the phenol-sulphuric acid method of Dubois et al. (1956) and for the presence of LPS by SDS-polyacrylamide gel electrophoresis in 15% gels and silver-periodate staining (see above). Alternative purification methods for the water-soluble LPS involving treatment of the aqueous extracts with heat-treated RNAase A (Sigma) to remove RNA were not used since this appeared to cause degradation of the LPS from 8530 and 8541, without affecting LPS from the wild type.

Oligosaccharides released from LPS by mild acid hydrolysis (1% acetic acid, 100°C, 3 h) were fractionated by column chromatography on Sephadex G-50. The column was equilibrated and eluted with 50 mM Tris-HCl pH 8 at a flow rate of 30 ml per h and 1.5-ml fractions were collected. Oligosaccharide elution was monitored with the phenol-sulphuric acid reagent (Dubois et al. 1956).

Monosaccharides were released from 1 mg of lyophilized LPS by hydrolysis in 1 ml of 2 M-trifluoroacetic acid at 105° C for 1.5 h. The hydrolysates were extracted with chloroform to remove fatty acids, lyophilized to remove the trifluoroacetic acid and taken up in 100 µl of water. Samples were fractionated by thin-layer chromatography on silica plates in ethyl acetate-pyridine-acetic acid-water (5:5:3:1 by volume) and sugars detected with *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bounias 1980). Alternatively chromatography was on cellulose plates in ethyl acetate-pyridine-water (8:2:1 by volume) and detection was with the alkaline silver reagent (Trevelyan et al. 1950).

DNA manipulation and sequence analysis.

Methods for preparation and restriction digestion of chromosomal DNA from bacteria are described in Parker et al. (1993). Separation of DNA fragments on agarose gels, Southern blotting, cloning procedures, and hybridization were performed essentially as described by Maniatis et al. (1982). DNA fragments were recovered from gels by centrifugation (Heery et al. 1990). Radioactive probes for hybridization were made by using a random priming kit (Megaprime, Amersham, UK). For the detection of homologues to *rfaX* and *rfaY*, probes were made by PCR using primers based on the sequence data in this paper and in Osbourn et al. (1990) with pIJ3122 as template DNA. Hybridizations were carried out under highly stringent conditions with final washes at 65°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1% (w/v) SDS. For sequence analysis, the following DNA fragments derived from pIJ3122::Tn*5* 8 were cloned into the pBluescript BS^- vector (Stratagene, La Jolla, CA): the 0.9-kb *Bam*HI-*Pst*I fragment of *Xanthomonas* DNA, the 1.06 kb *Pst*I-*Pst*I fragment which carries 680 bp of the inverted repeat of Tn*5* in addition to the *Xanthomonas* DNA, and the 2.7-kb *Bam*HI-*Hind*III fragment which carries 1,195 bp of the inverted repeat of Tn*5* in addition to the *Xanthomonas* DNA. Determination of the DNA sequence was done using the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems) with double-stranded DNA templates prepared as recommended by the manufacturer. Oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesiser. Nucleotide sequences were determined on an Applied Biosystems 373A DNA sequencer. Sequence homology searches were made with the BLAST program using BLOSUM 62. Sequence alignments were made with the algorithm PILEUP using a gap weight of 3.0 and a gap length weight of 0.1.

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