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In vivo cloning of the pectate lyase and cellulase genes of *Erwinia chrysanthemi*

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Using an RP4 plasmid which carries a mini-Mu prophage which allows it to integrate spontaneously random pieces of its host chromosome, we cloned *in vivo* at least some of the pectate lyase and cellulase genes of the *Erwinia chrysanthemi* strain B374. The RP4-prime plasmids were used to localize the cloned genes on the B374 chromosome by co-transposition mapping and to subclone most of the genes in a classic high copy number plasmid vector.

Key words: Erwinia chrysanthemi/pectate lyase/cellulase/gene cloning/RP4 plasmid

Introduction

The genus *Erwinia* consists essentially of a large series of phytopathogenic enterobacteria. Among them, the subspecies *Erwinia chrysanthemi*, which is responsible for the soft rot disease of plants in the field, in transit and in storage, presents the interesting property of secreting into the extracellular medium several enzymes such as pectinases (mostly pectate lyases), cellulases, protease(s) and DNAse(s) (for review, see Chatterjee and Starr, 1980). Secretion of pectinases and cellulases allows *Erwinia chrysanthemi* to digest the primary cell wall of plants and vegetables, both in culture and in conservation, and is in part responsible for the bacterial pathogenicity (Collmer *et al.*, 1982). Indeed mutants have been isolated which are defective for secretion, accumulate the enzymes in their periplasm and are not pathogenic (Andro *et al.*, 1984). The existence of such mutants strongly suggests that secretion proceeds through an active mechanism and not through a simple leakage of the enzymes across the outer membrane.

As with other enterobacteria, pectinolytic *Erwinia* are suitable for genetic analysis. They usually harbor no plasmid but many strains accept transferable plasmids such as RP4, and some are sensitive to bacteriophage Mu, two powerful tools for genetic analysis.

Separation by electrofocusing, of the proteins present in the supernatant of a culture of *E. chrysanthemi* strains followed by developing of the pectate lyase (PL; EC 4.2.2.2) activity directly on the gel, reveals the presence of five major pectic activities, two basic (called PLe and PLd), two neutral (PLc and PLb) and one acid (PLa) (Bertheau *et al.*, 1984). Therefore we expected these bacteria to contain more than one *pel* gene, which would explain why no *pel* mutants could be isolated so far. Cellulase(s) from *E. chrysanthemi* have not been extensively characterized. One strain (3665) was shown to produce one or two endoglucanases (Boyer *et al.*, 1984a, 1984b).

Here we describe the *in vivo* cloning, in *Escherichia coli*, of the pectate lyase (*pel*) genes of the Mu-sensitive *E. chrysanthemi* strain B374, using RP4::mini-Mu plasmids. Because they carry a mini-Mu with a functional transposase these plasmids can spontaneously integrate in their host chromosome by replicon fusion and, by subsequent mini-Mu mediated excision, generate R-prime plasmids which carry random pieces of the host chromosome (van Gijsegem and Toussaint, 1982). We also looked for R-prime plasmids carrying other genes specific to the genus *E. chrysanthemi* e.g., cellulase(s) and we found them as well (we shall use cellulase as a general term since at this point we do not know the type and number of enzymes involved). The R' *pel* plasmids were used to analyse both the pectate lyase activities which they encode and the unselected genes of B374 they carry. This allowed a further insight into the number of *pel* genes and their localization on the B374 chromosome.

Results

Cloning in E. coli of the pel and cel genes of B374 on RP4::mini-Mu.

B374/RP4::mini-Mu were mated at 35°C for 6–9 h with different polyauxotrophic derivatives of *E. coli* (KMBL241, AB2463 and CSH57). We first used *E. coli* recipients lysogenic for a lambdoid phage (λ or λi21) with the idea of being able to induce the transconjugants containing *pel* or *cel* genes to lyse, in case no pectate lyase or cellulase activity could be directly detected.

Table I. *E. coli* transconjugants analyzed

	pur$^+$			*ile*$^+$		
	Frequency of transposition of Pur$^+$[a]	Percentage of Pec$^+$	Percentage of Cel$^+$	Frequency of transposition of Ile$^+$[a]	Percentage of Cel$^+$	Percentage of Pec$^+$
Recipient: CSH57 rec$^+$						
Donor's plasmid:						
pULB113	2.1×10^{-7}	8%	<1%	1.3×10^{-7}	22%	1%
pULB110	4.5×10^{-8}	9%	<1%	4.2×10^{-8}	37%	1%
pULB108	5.5×10^{-8}	14%	<1%	2.5×10^{-7}	50%	0.5%
Recipient: CSH57 *recA*						
Donor's plasmid:						
pULB113	3.2×10^{-7}	7%	<0.2%	2.1×10^{-7}	0.2%	4%
pULB110	6.5×10^{-8}	14%	<1%	1.9×10^{-8}	<5%	7.5%
pULB108	2.5×10^{-7}	10%	<0.8%	3.5×10^{-7}	3%	13%

In all matings the donor was B374. It carried the RP4::mini-Mu indicated as the donor's plasmid. For each mating 2–5 independent experiments were carried out; the number of transconjugants tested in the different experiments ranged from 40 to 710 colonies. The numbers shown are the average calculated from the different experiments.

[a]The frequency of transposition is expressed as the number of Pur$^+$ (or Ile$^+$) clones *versus* the number of transconjugants which received the RP4::mini-Mu. The percentage of Pel$^+$ and Cel$^+$ transconjugants was calculated *versus* the number of Pur$^+$ or Ile$^+$ transconjugants.

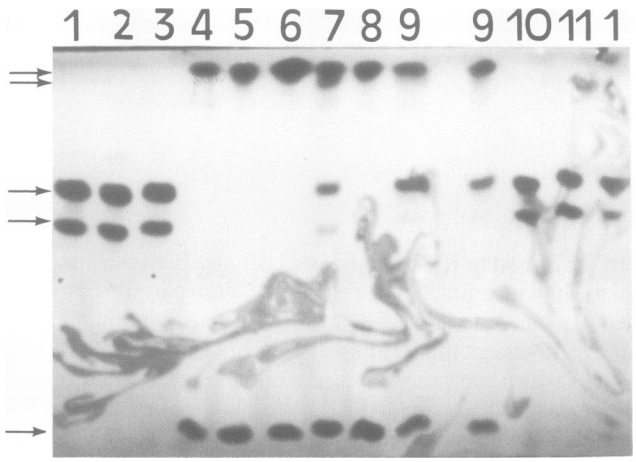

Fig. 1. Electrofocusing of supernatants of spheroplast prepared from different CSH57/R′ *pel* strains. **Slot 9:** B374 supernatant. The five major activities, two basic (PLd and PLe), two neutral (PLc and PLb) and one acid (PLa) are clearly visible. Note the difference in intensity between PLb and PLc. **Slots 1,2,3,10** and **11**: R′ *ile-pel*; pULB291, 290, 289, 327, 328. **Slot 4,5,6,7** and **8**: R′ *pur-pel*; pULB267, 266, 263, 262, 261. The different intensities between PLb and PLc are seen with the R′ *pur-pel* pULB262 but not with the R′ *ile-pel*. The samples of B374 and the R′ *pur-pel* were 20 times more concentrated than those of the R′ *ile-pel*.

This turned out not to be necessary and in further matings we used non-lysogenic *E. coli* recipients. From each mating, *E. coli* transconjugants were selected which had lost one or the other of their requirements. About 100 transconjugants of each type were replica-plated on PGTA and CMCA medium and after 24 h incubation they were tested for pectate lyase and cellulase activities. In the mating with CSH57 as a recipient ~9% of the Pur^+ and ~4.5% of the Ile^+ transconjugants had acquired a pectate lyase activity (Pel^+) and ~36% of the Ile^+ transconjugants had become cellulase positive (Cel^+). None of the Pel^+ clones was Cel^+ and *vice versa*, no Cel^+ clone was Pel^+, even among the Ile^+ transconjugants. These results are summarized in Table I.

Although no selection was applied for the presence of the plasmid, all the Pel^+ and Cel^+ clones had acquired all the resistance determinants of the RP4::mini-Mu used, as expected if they had received an R-prime plasmid. Analysis of the DNA content of several transconjugants of each type (five R′ *ile-pel*, 15 R′ *ile-cel* and 20 R′ *pur-pel*) confirmed that they all carried a plasmid larger than the original RP4::mini-Mu with sizes ranging from 119 to >300 kb (data not shown).

The presence of the *pel* genes on the R-prime plasmids was further suggested by the fact that they re-transferred at high frequency to a *recA* recipient without loosing their pectate lyase activity. However the R′ *ile-cel* did not re-transfer to either CSH57 *recA* or any other of the *ilv* mutants of *E. coli* tested as recipients; this will be discussed below.

Since *E. coli* usually does not export proteins further than the outer surface, we did not expect the pectate lyases or cellulases to be released in the culture medium by the Pel^+ and Cel^+ transconjugants. Thus, the detection of the pectate lyase and cellulase activities were most probably made possible by the lysis of a certain fraction of the cells during the long incubation on the tester plates. This was confirmed by the absence of activity in the supernatants of cultures of CSH57/R′ *pel* strains. However when spheroplasts of these strains were prepared, the pectate lyase activity was recovered in the supernatant suggesting that most of the activity was located in the *E. coli* periplasm (data not shown). The activity was assayed by adsorption at 235 nm (see Materials and methods). The R′ *pur-pel* displayed activities 10 – 100 times lower than the R′ *ile, pel*. As this assay measures the appearance of the unsaturated compounds formed by *trans*-elimination, it also confirms the PL nature of the enzymes encoded by the two types of R-prime plasmids.

Characterization of the pel genes

As mentioned above, 9% of the R-prime plasmids which carry the B374 *purE* gene, as well as 1 – 10% of those which have the *ile* gene(s), are Pel^+. To learn more about the number of genes involved in the Pel^+ phenotype, spheroplasts were prepared from different CSH57/R′ (five R′ *pur-pel* and five R′ *ile-pel*) and their supernatants were run on an electrofocusing gel. Figure 1 shows that the R′ *pur-pel* plasmids carry either the basic (PLe and PLd) and the acid (PLa) but not the neutral activities (pULB261, 263, 266 and 267) or the five major activities (pULB262). The R′ *ile-pel* carry the two neutral pectate lyases PLb and PLc (pULB289, 291, 292, 327, 328). This suggests, in agreement with what was found by Kotoujansky *et al.* for another *E. chrysanthemi* strain, (see accompanying paper), that on the B374 chromosome, the genes encoding the polypeptide(s) involved in the basic and acid activities are strongly linked as are those involved in the neutral activities, the two sets of genes being in addition more loosely linked to each other. As mentioned earlier, the pectate lyase activities of the R′ *ile-pel* are higher than those of the R′ *pur-pel*. This difference might be due to the presence of one or more regulatory genes on only some of the R-prime plasmids.

Several R-prime plasmids were characterized physically. The DNA of four R′ *pur-pel* plasmids was purified, digested with *Pst*I, electrophoresed on 0.7% agarose gels, the fragments transferred by blotting on nitrocellulose filters and hybridized with a ^{32}P-labelled Mu DNA probe (see Figure 2). The results of the hybridizations show that the four plasmids (pULB261, 262, 263 and 267, Figure 2A) carry six fragments which contain Mu DNA (two of which are identical and thus form a doublet), as expected for two mini-Mus flanking a single B374 chromosomal fragment, strongly suggesting that the genes found linked on the plasmids are also linked on the B374 chromosome. pULB262, which carries the five major activities, has ~130 kb of host DNA. Thus all the *pel* genes are most probably located on 3 min of the B374 chromosome. Four R′ *ile-pel* were analysed in the same way as the R′ *pur-pel*. However, because their genetic analysis had revealed their tendency to loose the *pel* genes, total DNA was extracted from the four corresponding CSH57/R′ *ile-pel* strains using cultures which had been re-checked for the expression of pectate lyases just before use. The total DNA was digested with *Pst*I and tested simultaneously with the R-prime plasmid DNA digests. As seen in Figure 2, the pattern of hybridization of the R′ *ile-pel* with ^{32}P-labelled Mu DNA is slightly more complex than those of the R′ *pur-pel*. However the major hybridizing bands are in most cases similar in the purified plasmid DNA and the total DNA, suggesting that no major rearrangement(s) had occured on the purified plasmids. However, the hybridization patterns are not as consistent with the expected pattern as were those of the R′ *pur-pel*. pULB291 most probably carries more than one segment of host DNA since it displays more than five bands hybridizing Mu, thus more than two mini-Mus. In pUBL290 and pUBL327 there are five bands hybridizing Mu, as expected, and pUBL291 shows only three or four such bands, suggesting that some of them might actually be doublets which both contain Mu DNA. The more complex behavior of the R′ *ile-pel* could be explained if in B374, the

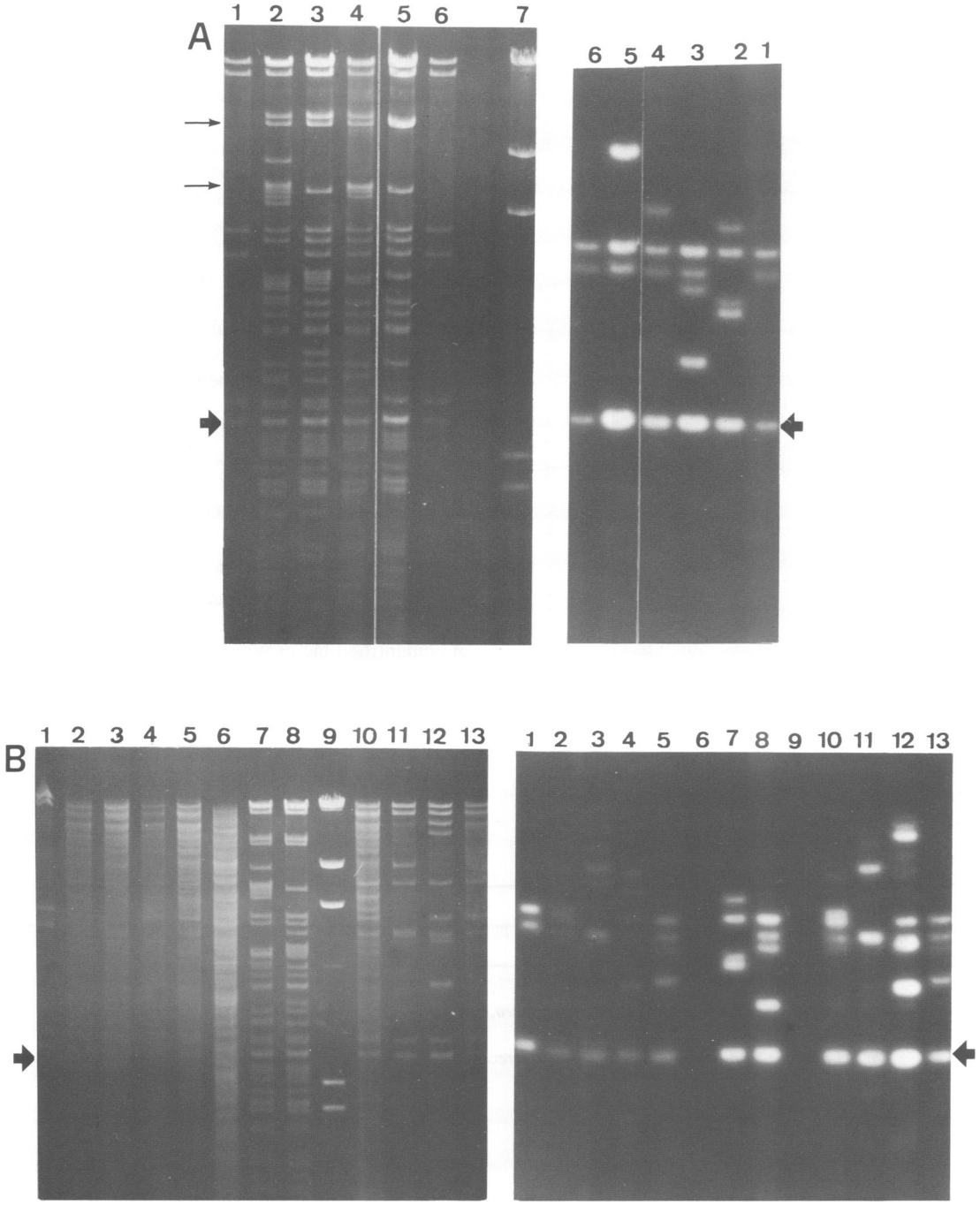

Fig. 2. 1μg of each DNA was digested with *Pst*I and run on a 0.7% agarose gel for 16 h at 20 mA. λ DNA digested with *Hind*III served as size marker. The fragments were measured on a Hewlett-Packard Digitizer connected to an HP9815A calculator. The fragments were blotted onto nitrocellulose filters and hybridized to labelled Mu DNA as described in Materials and methods. The thick arrows point towards the Mu3A-RP4 fusion fragments (for reference see van Gijsegem and Toussaint 1982). **(A)** R′ *pur-pel*; **slot 1** and **6**: pULB113; **slot 2–5:** pULB261, 262, 263 and 267; **slot 7:** λ digested with *Hind*III. One of the Mu3A-RP4 fusion fragments of pULB261 suffered a deletion. pULB263 and 267 show their two new Mu3A-B374 DNA junction fragments as a doublet as clearly seen on the agarose gel and pointed out by the thin arrows. **(B)** R′ *ile-pel*; **slot 1:** pULB113; **slot 2–5:** total DNA from CSH57 carrying pULB289, 290, 293 and 327, respectively. **Slot 6:** B374 DNA; **slot 7:** pULB261; **slot 8:** pULB262; **slot 9:** λ digested with *Hind*III; **10:** pULB289; **11:** pULB290; **12:** pULB291; **13:** pULB327.

chromosomal origin of replication, *oriC*, is located very close to *ile* as it is in *E. coli*. In that case most R′ *ile* could carry *oriC*, and the presence of that new replication origin might provoke some instability in the plasmids, leading to rearrangements in their structure and consequently to more complex digestion patterns. The DNA of the pULB262 was digested with either *Eco*RI, *Hind*III, *Bam*HI or *Pst*I and the fragments were ligated to pBR322 DNA cut with the same enzyme. After transformation in the *E. coli* strain DS410, the Pel$^+$ clones were screened on PGTA medium. They were purified and grown in minimal medium supplemented with galacturonate and polygalacturonate. Spheroplasts were prepared and their supernatants run by electrofocusing. The three *pel*$^+$ clones resulting from the *Bam*HI digest as well as one from the *Pst*I digest all contain PLe, PLd and PLa, while the eight clones resulting from *Eco*RI digest express only PLd. The 12 clones resulting from digestion with *Hind*III express on-

ly PLe and PLa. The PLc and PLb could not be recovered from any of the above digests. The electrofocusing of one derivative of each type is shown in Figure 3.

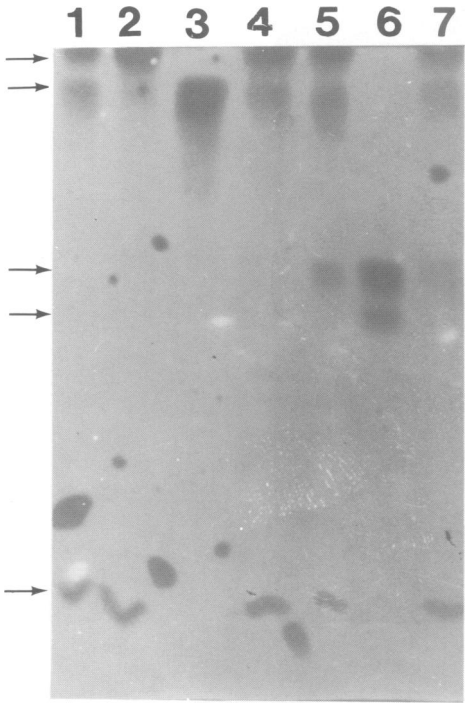

Fig. 3. Electrofocusing of the supernatants of the *E. coli* strain DS410 carrying different pBR322::*pel* plasmids resulting from cloning of *Bam*HI (**slot 1**), *Hind*III (**slot 2**), *Eco*RI (**slot 3**), *Pst*I (**slot 4**). Controls: supernatants of CSH57/pULB262 (**slot 5**), CSH57/pULB327 (**slot 6**) and B374 (**slot 7**).

Characterization of the R' cel plasmids

Since the R' *ile-cel* isolated in CSH57 could not be re-transferred to other *E. coli* strains we could not directly verify the linkage between *cel* and *ile*. We found previously that the *kdgK*, *uxuA* and *uxuB* genes are linked to the *ile* marker on the B374 chromosome (van Gijsegem *et al.*, 1985). To check the existence of a linkage between *ile* and *cel* we tested the cellulase activity of several previously characterized R-prime plasmids derived from pULB113 and carrying *kdgK* and (or) *uxuA-B* (three R' *kdgK-uxuA-uxuB*, pULB119, 121 and 122, and two R' *uxuA-uxuB*, pULB123 and 124). All but pULB124 turned out to be positive. Those five plasmids had been previously shown to carry a unique B374 chromosomal DNA fragment (van Gijsegem and Toussaint, 1983). These observations confirm the existence of a linkage between *cel* and the *kdgK* and *uxu* genes, and therefore of *ile*, with *cel*.

pULB121 DNA was digested with *Eco*RI and ligated to pBR322. This allowed us to recover the cellulase activity on a 13-kb long *Eco*RI fragment which is currently further analyzed (Verhemeldonk, in preparation).

Genetic analysis of the ile region of the B374 chromosome

As mentioned earlier, the R' *ile-cel* could not be transferred to any of the *E. coli ile* strains we tested (see *ile* strains in Table II). We suppose that B374 carries, between its *ile* and *cel* genes, an unidentified factor which is lethal for most *E. coli* strains but not for CSH57. This factor is obviously not present on the R' *ile-pel*, since they readily transfer among *E. coli* recipients. This allowed us to further analyze the genetic organisation of the *pel* genes in the *ile* region of the B374 chromosome. B374/pULB110 was mated with different *E. coli* recipients carrying mutations in genes whose equivalent were known to map around the *ile* marker in B374 (*ile*, *met*B, *cya*). Different types of R-prime

Table II. Bacterial strains and plasmids

	Characters	References
E. coli strains		
CSH57	*leu purE,trp,his, argG,ilv, met,mtl,gal,lacY,xyl,ara,malts,rpsL*	Miller (1972)
RH5473	same as CSH57 but srl::Tn*10*,*recA*,Mu^R,*rpsE*	This laboratory
KMBL241	*thr,leu,pyrF,his,thyA,argG,thi,ilvA,lacY,tonA,tsx,recA*	van de Putte *et al.* (1966)
AB1157	*thr,leu,pro,his,thi,argB,ara,xyl,mtl,gal,lacY,rpsL*	Howard-Flanders *et al.* (1964)
CT110	*kdgR,kdgK,metB,ilv,lac,gal,mal,mtl*	Pouyssegur and Lagarde (1973)
KL584	HfrC, Δ(*pro,lac*) XIII,*cysG,met*	B. Low
TP7860	*his*, Δ*cya*	A. Danchin
TP7860 *ilv*::Tn*5*	P1 transduction of *ilv*::Tn*5* in TP7860	This laboratory
DS410	*minA,minB,rpsL,sup*	Dougan and Sherratt (1977)
RH5768	Δ(*pro,lac*)*purE,trp,his,argG,ilv*::Tn*5*,*malts,gal,rpsL*	P1 transduction of *ilv*::Tn*5*, Δcya, from TP7860 *ilv*::Tn*5* into a Leu$^+$, Δ(*pro,lac*) recombinant between CSH57 and KL584
Erwinia strains		
B374	*Erwinia chrysanthemi*	Hamon and Peron (1961)
3937J	*Erwinia chrysanthemi*	Kotoujansky *et al.* (1982)
Plasmids		
RP4	Tc^R Ap^R Kn^R	Saunders and Grindsted (1972)
pULB113	RP4::Mu3A	Van Gijsegem and Toussaint (1982)
pULB110	RP4Kn::Mu3A	This laboratory
PRM5	RP1*ts*	Robinson *et al.* (1980)
pULB108	PRM5::MU3A,ΔTc	This laboratory
Phages		
λ*i*21*c*$^+$		Liedke-Kulke and Kaiser (1967)
Mu3A	mini-Mu *A*$^+$ *B*$^-$	Résibois *et al.* (1981)

The nomenclature used is that of Bachmann (1983) except for genes specific to *Erwinia-pel* stands for pectate lyase structural genes, Pel for the pectate lyase phenotype, *cel* for cellulase structural genes and Cel for cellulase phenotype.

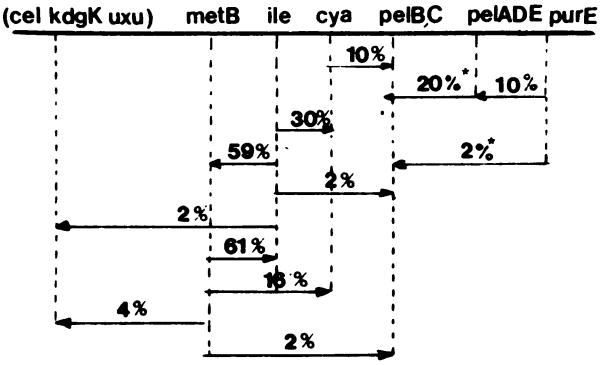

Fig. 4. Linkage map in the *ile* region of the B374 chromosome. The experiments leading to the construction of this map are described in Materials and methods. The arrows point from the selected towards the unselected markers. The numbers represent the percentage of co-transfer of the unselected marker with the selected one. The frequency of co-transposition of Cel with *ile* indicated here which was obtained using CT110 as a recipient is much lower (2% compared with ~37%) than that shown in Table I where CSH57 was the *E. coli* recipient. We suppose that this is due to the presence of an unidentified factor, located between Cel and *ile* on the B374 chromosome, which is lethal for CT110 but not for CSH57 (see text for more discussion). Frequences of co-transposition inferred from the presence of one R'*pur*, *pelA*, *pelB*, *pelC*, *pelD*, *pelE* among five R'*pur*, *pel* analyzed by electrofocusing (see Figure 1).

plasmids were selected and screened genetically for the presence of unselected markers to measure their frequencies of co-transposition. These results and the genetic map they allow us to draw are presented in Figure 4.

The existence of pULB262 suggests that the linkage between *purE* and *pelB, pelC* is equivalent to the linkage between *ile* and these two *pel* genes. However we cannot exclude at this point that there are actually two copies of the *pelB-C* genes on the B374 chromosome, one linked to *purE* and the other to *ile*. No common *Pst*I restriction fragments can be seen between pULB262 and the R' *ile, pel* which all express PLb and PLc (see Figure 2B). However since the *PelB* and *PelC* genes are co-transposed at only 1–5% with *ile* and *purE*, respectively, they are very likely to be located at the end of the chromosomal DNA segment on the R-prime plasmids thus in one of the mini-Mu chromosomal DNA fusion fragments which have no reason to be of identical size in different R-prime plasmids. We are currently trying to map the *purE* gene of B374 to see whether it actually resides in the neighbourhood of *ile*.

Discussion

By simply mating *Erwinia* donors which carry an RP4::mini-Mu plasmid with a polyauxotrophic *E. coli* recipient which has seven requirements, and selecting *E. coli* transconjugants complemented for one of their auxotrophy, we could isolate R-prime plasmids which carry B374 genes encoding pectate lyases and cellulases. These genes are thus readily expressed in *E. coli*.

It is likely that we recovered all the existing pectate lyase genes since (i) we have recovered the five major pectic activities detected after electrofocusing of the proteins contained in the supernatant of a culture of B374 (ii) Kotoujansky *et al.* (accompanying paper) recovered the same type of clones, showing either the neutral or the basic and acid activities, in their clone bank of another *E. chrysanthemi* strain (3937j) which shows the same electrofocusing pattern as B374. It is however possible that other *pel* genes exist in B374 which either cannot be expressed in *E. coli* or are duplications of the genes we cloned and therefore would have escaped our detection.

It is at this point difficult to estimate whether the method of *in vivo* co-cloning which we used could be widely applied to bacterial species where the RP4::mini-Mu is functional or whether we were dealing with a particularly favorable case, since all the genes we wanted to recover turned out to be closely linked on the B374 chromosome. We know that R-prime plasmids derived from RP4::mini-Mu can be at least 300 kb long, but we have so far only a preliminary estimation of their average size; therefore we cannot calculate what should be the optimal number of markers to be used in the recipient to insure the recovery of a set of R-prime plasmids representative of the whole donor chromosome. Moreover the existence of lethal factors such as the one we found associated with the *cel* genes, would in some cases impose additional constraints on the selective markers to be used in the recipient. However such factors would raise problems with any other cloning technique as well.

The fact that our method provides the genes of interest on a broad host range and self-transmissible plasmid, presents several advantages; one is the possibility of transferring the R-prime plasmids in different bacteria carrying a variety of markers (see Figure 4) allowing us to map the unselected genes present on the plasmid by their frequency of co-transposition with the selected markers; a second is that the plasmids can also be transferred to bacteria of genera other than both the donor or the recipient – some of the R' *pel* plasmids described here have been transferred in *Myxococcus xanthus* where they were found to express the *Erwinia pel* genes (Guespin-Michel, personal communication).

We were surprised to recover the *pel* genes corresponding to PLc and PLb as these were not found in a gene bank prepared from B374 DNA on the cosmid vector pMM33 (Reverchon *et al.*, in preparation). This could be related to the fact that we could not subclone these genes on pBR322; perhaps, when present on a high copy number plasmid (and therefore produced in large amount) they are lethal to the *E. coli* host.

Recently Keen *et al.* (1984) reported the cloning on a cosmid vector, in *E. coli*, of the two PL activities they identified in *E. chrysanthemi* EC16. They concluded to the existence of two *pel* genes in that strain. At this point it is not possible to correlate their results with ours. Indeed their electrofocusing technique differs from ours and revealed only two PL of pI 8.8 and 9.8, respectively. Collmer (personal communication) has shown that in *E. chrysanthemi* CUCPB1237, two PL of pI 8.3 and 9.3 actually correspond to two pairs of PL of the same pI and mol. wt. which would correspond to our PLb and c and PLd and e, respectively. Therefore there is a strong possibility that in EC16 the situation is similar and more complex than presently reported.

The cloning of the B374 *pel* genes has allowed a first insight into their number and genetic organization. Because B374 produces five major pectate lyase activities, we tend to believe that there are at least five genes involved in their synthesis. From the different types of plasmids we isolated we now know that at least (i) PLd, (ii) PLc and b and (iii) PLe and a correspond to different genes. Moreover the wide difference in pI between PLe and PLa makes it unlikely that they are encoded by the same gene. Therefore we are fairly confident we identified at least four genes involved in PL synthesis.

Moreover the PLb and maybe PLc activities displayed by the R' *ile-pel* are higher than those displayed by the R' *pur-pel* suggesting that the PL on the R' *ile-pel* might be derepressed and thus that a negative regulatory gene might be present on the R' *pur-pel*.

Genetical analysis of the R' *cel* and R' *pel* showed that the *kdgK, uxuA-uxuB, cel* genes and those encoding PLb-PLc are

all linked to *ile* but to different extents. We did not find any R-prime plasmid expressing PLb-PLc and Cel, from which we conclude that *ile* is located between the genes encoding PLb-PLc and the *cel* gene(s). All the R-prime plasmids which carry *ile*, PLb and PLc also carry *cya* placing *cya* between *ile* and PLb-PLc. *met* was found to be strongly linked to *ile* and most probably located between *cel* and *ile*. Finally PLa, d and f are linked to *purE*. One R′ *pur-pel* out of five also carries the five PLs but none did carry *cya*. All these markers are therefore most probably arranged in the order (*kdgK, uxuA-uxuB, cel*)-*met-ile-cya-*(*pelB, pelC*) (*pelA, pelD, pelE*), *purE* (where *pelA*−*E* would be the genes corresponding to PLa−e, respectively). Physical analysis of the R′ *cel* and R′ *pel* showed that most of them carry a unique segment of B374 chromosomal DNA flanked by a direct repeat of mini-Mu confirming the linkage deduced from genetic analysis.

In order to correlate our results with those presented by Kotoujansky *et al.* in the accompanying paper we attempted to clone the pectate lyase genes of their strain, 3937j, using the same conditions as those used for B374. The mating between 3937j/pULB113 and CSH57 provided R′ *purE* at about the same frequency as the matings with B374/pULB113 as donor but no R′ *pur-pel* plasmid could be found (<0.3% data not shown). Moreover the R′ *pel* pULB267 complements the *pur*3 mutation of 3937j which has been located very far away from the *ile* locus in that strain (Kotoujansky *et al.*, 1982). Therefore there is a strong possibility that the chromosomes of these two strains of the same *Erwinia* subspecies are quite differently organized.

The presence of more than one *pel* gene has so far prevented the isolation of *pel* mutants of *E. chrysanthemi*. Such mutants will now be easy to isolate from the cloned genes. Moreover the physical characterization of the part of the gene encoding for the NH_2-terminal portion of the proteins should bring further information about the mechanism of their secretion.

Materials and methods

Bacteria were grown in L broth (Miller, 1972), titrated on L agar (L broth with 1.2% agar) and diluted in 10 mM $MgSO_4$. Minimal medium was 132 Ceria (Glansdorff, 1965). When required, antibiotics were added at a concentration of 25 μg/ml for ampicillin (Ap), tetracycline (Tc) and kanamycin (Kn) and 100 μg/ml for streptomycin (Sm) and spectinomycin (Sp). Sugars were added at a final concentration of 0.2%, casamino acids of 0.1%, amino acids of 40 μg/ml, uracil and adenine of 50 μg/ml and thymine of 100 μg/ml. PGTA medium (Bertheau *et al.*, 1984) was used to detect pectate lyase activity and CMCA medium (Wood, 1981) to screen for cellulase activity.

Tryptone, yeast extract and agar were purchased from Gibco, agarose from BRL, sodium polygalacturonate from Sigma (85−90% pure), carboxymethylcellulose from Serva.

Pectinase activities were revealed after at least 24 h growth at 35°C on PGTA medium with 10 ml 7.5% copper acetate (Bertheau *et al.*, 1984). Cellulase activities were revealed by the congo red method (Wood, 1971) after the same incubation time.

Matings were performed by spotting 0.01−0.05 ml of overnight cultures of donor and recipient strains on L agar, incubating the plates for 6−9 h at 35°C, resuspending the mating mixtures in 1 ml 10 mM $MgSO_4$ and spreading 0.1 ml aliquots on selective media. Plates were incubated at 35°C for 24−48 h. Transconjugants were usually tested directly for pectate lyase and cellulase activity by replica-plating on PGTA and CMCA medium containing Str to prevent growth of the *Erwinia* donor. Mapping by co-transposition was described by Faelen and Toussaint (1976). The *pel* genes were mapped *versus* different markers in the *ile* region of the B374 chromosome as follows; (i) B374/pULB113 was mated with a Δ*cya* derivative of CSH57 which is *mal*ts. Mal^+ transconjugants were selected at 42°C and tested for the presence of pectinase activity. (ii) B374/pUBL113 was mated with CSH57 (*purE*). Pur^+ transconjugants were selected and tested for their pectinase activity. (iii) B374/pULB110 was mated with the *E. coli* strain CT110 which is *ilv, metB*. *E. coli* transconjugants were selected which received either an R′ *ile* (Ile^+) or an R′ *metB* (Met^+). 100 Ile^+ and 100 Met^+ transconjugants were tested for the presence of the unselected markers (a) Met^+ and Ile^+, respectively (b) Cel^+, (c) Pec^+. The plasmids were further transferred in a Δ*cya E. coli* and the transconjugants were tested for the acquisition of a Cya^+ allele.

Mini-scale plasmid DNA preparations were made according to either Kado and Liu (1981) or Birnboin and Doly (1979), large-scale preparations according to Betlach *et al.* (1976). Digestion of the DNA with restriction enzymes, separation of the restriction fragments on agarose gels and subcloning of the R-prime plasmid restriction fragments in pBR322 were as described by Maniatis *et al.* (1982). The Mu DNA was purified from a Mu*cts62* lysate grown upon induction of a *Proteus mirabilis* lysogen, to avoid hybridization of the Mu variable end with *E. coli* and *E. chrysanthemi* chromosomal DNA. Restriction fragments were transferred onto nitrocellulose filters as described by Southern (1975) and hybridized to a ^{32}P-labelled Mu DNA probe according to Maniatis *et al.* (1982). Electrofocusing was as described by Bertheau *et al.* (1984) and spheroplasts were prepared as described by Andro *et al.* (1984). Pectate lyase was assayed by following the increase in adsorption at 235 nm, at 37°C after adding 200 μl of periplasmic fraction of the *E. coli*/R′ *pel* to 800 μl of reaction mixture (0.125% PGA, 1 mM $CaCl_2$, 100 mM Tris pH 8.6).

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