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A Broad-Host-Range Expression Vector Based on the p_L Promoter of Coliphage λ: Regulated Synthesis of Human Interleukin 2 in *Erwinia* and *Serratia* Species

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We report the construction of a broad-host-range expression vector based on an RSF1010-derived replicon. The vector carries the strong leftward promoter (p_L) of coliphage λ as well as the *c*I857 allele, which codes for a thermolabile repressor protein. The coding region of mature human interleukin 2, which is preceded by the *ner* ribosome binding site of phage Mu, was cloned downstream from the p_L promoter. The plasmid was introduced into *Erwinia* and *Serratia* species by means of mobilization. Heat-inducible synthesis of interleukin 2 protein was obtained, showing that the p_L promoter is functional in these genera. As in *Escherichia coli*, the bulk of the overproduced protein was present in an insoluble form.

In recent years much attention has been paid to the development of gene cloning and expression systems in bacteria other than the widely used *Escherichia coli* (13, 22, 35). Many gram-negative bacteria, some of which are of medical or agricultural importance (27, 38), possess a broad diversity of metabolic activities and might therefore be promising hosts for cloning and expression studies. Indeed, some genes can only be functionally expressed in a particular physiological background, e.g., when an enzyme is integrated into a specific biochemical pathway (21, 33) or when the base composition of the foreign gene differs widely from that of the host genome. It has been suggested that the weak expression of *Pseudomonas* genes in *E. coli* may be attributed to the unusual codon preference of *Pseudomonas* species (28). Therefore, there is a need for expression vectors that are adapted to the host of interest. The most versatile of these are likely to be based on broad-host-range plasmids, which can be stably inherited over a wide range of gram-negative species (2).

We describe the cloning of the strong leftward promoter (p_L) of coliphage λ, together with the *c*I repressor gene, onto a derivative of the broad-host-range plasmid RSF1010 (19). This plasmid was introduced into three other enteric bacteria: *Serratia marcescens*, *Erwinia chrysanthemi*, and *Erwinia carotovora* subsp. *carotovora*. By obtaining controlled expression of cloned human interleukin 2 (IL-2), we demonstrate that the p_L promoter is functional in these organisms and that its activity is regulated by the *c*I repressor as in the natural host *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Unless otherwise mentioned, bacterial cultures were grown at 28°C in LB medium (1% tryptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract [Difco], 0.5% NaCl). Antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; carbenicillin, 100 μg/ml; tetracycline, 10 μg/ml.

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Transformation and conjugation procedures. *E. coli* strains were transformed as described previously (31). Plasmid DNA was introduced into *Serratia* and *Erwinia* species by means of conjugational transfer by using pRK2013 (16) or pRK2073 (26) as mobilizing plasmids. Conjugation was performed on solid media or in liquid culture by mixing appropriate dilutions of donor and acceptor strains (usually in a 1:10 ratio). The mixed culture was incubated for 5 to 6 h at 28°C before it was plated onto selective agar plates. *Erwinia* and *Serratia* exconjugants were detected by using appropriate selection techniques (see below).

DNA manipulations. Plasmid DNA was isolated from 3-ml cultures by the sodium dodecyl sulfate-sodium hydroxide lysis procedure (5). Restriction enzymes, purchased from New England Biolabs (Beverly, Mass.) or Boehringer GmbH (Mannheim, Federal Republic of Germany) were used as recommended by the suppliers. Protruding ends were filled in by using the Klenow fragment of DNA polymerase I, essentially as described previously (37). T4 DNA ligase was purified from K12ΔH1Δtrp(pPLc28lig8) (32). Ligation of fragments was performed as described previously (31).

Detection of induced proteins. (i) Induction procedure. Cells were grown overnight in selective LB medium at 28°C. The culture was then diluted 200-fold in 20 ml of fresh medium. When a density of 5×10^8 cells per ml was reached, 10-ml fractions were shifted to 42°C and incubated for another 2-h period. The cells were then pelleted, suspended in sample buffer, and boiled for 5 min. Electrophoresis on 15% polyacrylamide gels was carried out as described by Laemmli (23). The gels were fixed in 10% trichloroacetic acid. Proteins were stained by using 0.05% Serva Blue R (Serva, Heidelberg, Federal Republic of Germany) in 30% methanol–7% acetic acid.

(ii) Western blotting. Antibodies directed against human IL-2 were raised in rabbits that were injected with purified IL-2, which was obtained from the overproducing *E. coli* K12ΔH1Δtrp(pPLcMuHIL201) strain (12). To reduce nonspecific background, the serum was preadsorbed with a lysate of *E. coli* K12ΔH1Δtrp. For immunological characterization the proteins were electrophoretically blotted onto a nitrocellulose filter (pore diameter, 0.45 μm; Millipore

TABLE 1. Bacterial strains used in this study

Strain	Genetic characters	Reference
Escherichia coli		
HB101	*hsdS20 recA13 ara14 proA2 lacY1 galK2 rspL20 xyl-5 mtl-1 supE44*	7, 8
MC1061	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK* r_K^- m_K^+ Sm^r	11
K12ΔH1Δtrp	Sm^r *lacZ*(Am) Δ(*bio-uvrB*) Δ*trpEA2* (λ *N*am7am53 *c*I857 ΔH1)	4
Serratia marcescens W831	Deficient in exocellular protease	14
Erwinia chrysanthemi NCPPB 2309		
Erwinia carotovora subsp. *carotovora* NCPPB 312		

Corp., Bedford, Mass.) in transfer solution (25 mM Tris hydrochloride [pH 8.3], 192 mM glycine, 20% methanol) during a 2-h period (41). To block any remaining free binding sites, the filter was saturated with protein by using a 2.5% nonfat milk powder solution (in 0.01 M NaH_2PO_4[pH 7.5], 0.15 M NaCl, 0.2% Triton X-100). Incubation with the antibody was performed at room temperature for 1 h. Excess unbound antibody was removed by washing. The filter was then treated with a goat anti-rabbit antibody conjugated with alkaline phosphatase (Promega Biotec, Madison, Wis.) Following a second incubation-rinsing period, the bands were visualized in 0.1 M Tris hydrochloride (pH 9.5)–0.1 M NaCl–50 mM $MgCl_2$ containing 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as colored substrates (6, 24). The enzymatic reaction was ended after 10 to 60 min with 25 mM Tris hydrochloride (pH 7.5)–5 mM EDTA.

(iii) Biological assay for IL-2. To test for biological activity of bacterially synthesized human IL-2, the different strains were grown at 28°C and induced at 42°C. Cells were opened by sonication, cell debris was spun down (16,000 rpm, 30 min), and the supernatant was passed through a filter (pore diameter, 0.2 μm; Millipore). These extracts were then assayed for T-cell growth-stimulating activity essentially as described previously (12), except that an IL-2-dependent cell line was used (17). In this assay only the IL-2 that was soluble in the lysate was measured.

RESULTS

Construction of expression vectors. The construction of the expression vectors used in this study is outlined in Fig. 1. First, we introduced the p_L promoter of coliphage λ into plasmid pKT240. This plasmid is derived from RSF1010 and carries functional resistance genes for ampicillin and kanamycin (from Tn*A* and Tn*903*, respectively) (3). An *Eco*RI-*Hin*dIII fragment containing the amino-terminal part of the Km^r gene was excised from pKT240 and replaced with an *Eco*RI-*Hin*dIII fragment from pPLa832 (31). A representative plasmid was designated pPL1010. Second, we introduced the *c*I gene, coding for the λ repressor protein, into plasmid pPL1010. The *c*I857 mutant allele, encoding a temperature-sensitive repressor, was obtained from phage λ *c*I857 *S*am7 as a *Bgl*II-*Pst*I fragment (39) and was cloned between the *Bam*HI and *Pst*I sites of pPL1010, replacing the Ap^r gene. A representative plasmid was designated pPLGN1.

TABLE 2. Plasmids used in this study

Plasmid	Genetic characters	Reference
pKT240	Mob^+ Km^r Ap^r RSF1010 origin	3
pRK2013	Tra^+ Mob^+ (RK2) Km^r ColE1 origin	13
pRK2073	Tra^+ Mob^+(RK2) Km::Tn*7* ColE1 origin	26
pPL1010	Mob^+ Km^r Ap^r p_L^+ RSF1010 origin	This study
pPLGN1	Mob^+ Km^r p_L^+ *c*I857$^+$ RSF1010 origin	This study
pPLGN1HIL2	Mob^+ Km^r p_L^+ *c*I857$^+$ IL2$^+$ RSF1010 origin	This study

In a subsequent step the coding region of the human IL-2 gene was cloned downstream from the p_L promoter in pPLGN1. The IL-2 fragment was obtained from pPLcMuHIL201 (12), in which the gene is fused in frame to a ribosome binding site derived from the *ner* gene of phage Mu. Plasmid pPLcMuHIL201 was opened at its unique *Bam*HI site, the single-stranded ends were filled in by using Klenow polymerase, and the vector was subsequently cleaved with *Eco*RI. This fragment was then inserted between the unique *Eco*RI and *Pvu*II sites of pPLGN1. A representative plasmid was designated pPLGN1HIL2.

Expression in *E. coli*. To test the functionality of the newly constructed vectors, strain MC1061(pPLGN1HIL2) was induced at 42°C for 2 h and monitored for the synthesis of IL-2 protein. A prominent new band, corresponding in molecular weight to IL-2, was synthesized (Fig. 2). Surprisingly, the expression level was much higher than the level obtained with pPLcMuHIL201 in strain MC1061(pcI857). The reason for this difference remains unclear. Both plasmids contain the same transcriptional and translational control elements and display comparable copy numbers in the host strain used (data not shown).

Expression in *Erwinia* and *Serratia* species. (i) Introduction of the vectors into *Erwinia* and *Serratia* species. Attempts at transformation of the *Erwinia* and *Serratia* strains were unsuccessful, despite the fact that several procedures were tested. We then decided to introduce the vectors by means of conjugational transfer by using pRK2013 (16) or pRK2073 (26) as mobilizing plasmids. Therefore, conditions for the efficient transfer and selection of exconjugants were established by using pKT240, as this plasmid was known to have retained the broad-host-range replication properties of RSF1010 (3). A culture of *E. coli* HB101(pRK2013, pKT240) was incubated together with the acceptor strain, either *Erwinia* or *Serratia* species (see above).

S. marcescens W831 was shown to be resistant to tetracycline (10 μg/ml). Therefore, exconjugants were selected on agar plates for their resistance against tetracycline (to select against the *E. coli* donor), kanamycin, and carbenicillin (resistance encoded by the incoming plasmid). Plates were incubated at 28°C, and red colonies appeared overnight.

Neither *E. chrysanthemi* nor *E. carotovora* showed resistance to any of a large number of antibiotics tested (data not shown). In this case, counterselection of the donor bacteria was achieved by infection with the *E. coli*-specific phage λ*vir*. Hence, the growth medium was supplemented with 0.2% maltose–10 mM $MgCl_2$. The phage was added at a multiplicity of 10, and the culture was further incubated under vigorous aeration until total lysis of the *E. coli* bacteria

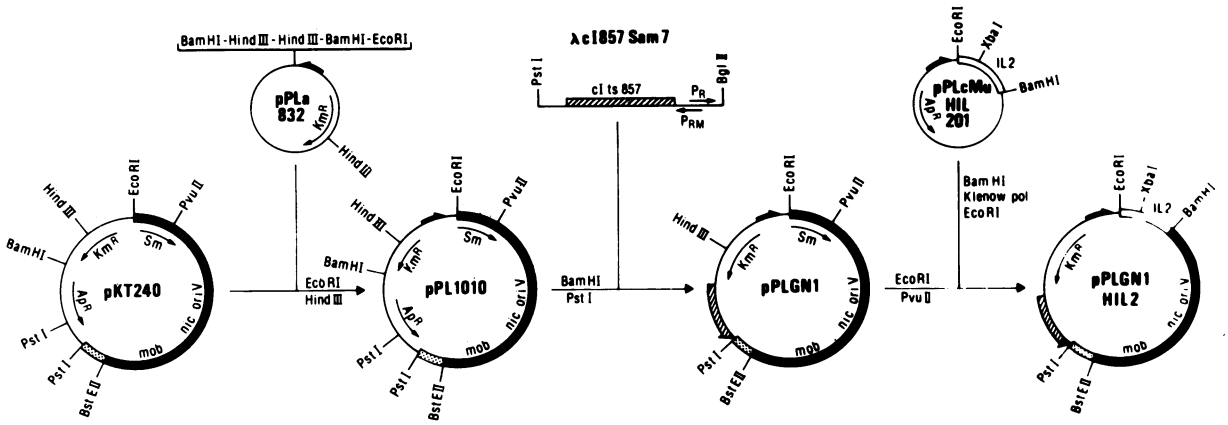

FIG. 1. Construction scheme of the broad-host-range expression vectors. The heavy line represents sequences derived from RSF1010; the dotted line represents a sequence presumably derived from RSF1010. The location and direction of transcription of the p_L promoter is indicated by a heavy arrow. The arrows represent the direction of transcription. Abbreviations: ori V, origin of vegetative replication; nic, relaxation nick site; mob, mobilization functions; Km, kanamycin; Ap, ampicillin; Sm, streptomycin.

occurred (after 5 to 6 h). The mixed culture was then plated onto selective plates and incubated at 28°C. In most cases we obtained less than one *E. coli* colony per 100 exconjugants. The acceptor colonies were investigated for β-lactamase production by using Ampscreen filters (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) impregnated with the chromogenic cephalosporin Padac (34). Conjugants induced a pronounced color shift from violet to yellow within 30 s, whereas the plasmidless control colonies did not.

The expression vectors pPLGN1 and pPLGN1HIL2 could be mobilized into *Serratia* and *Erwinia* species in the same way described above by using pRK2073 as the transfer-providing plasmid (tra^+). The presence and physical intactness of pPLGN1HIL2 in *Erwinia* species was further confirmed through restriction analysis of isolated plasmid DNA (data not shown). In the case of the *Serratia* strains, however, we failed to obtain sufficient plasmid DNA to allow its physical analysis. Presumably, the excretion of potent DNases interferes with the isolation of large amounts of intact plasmid DNA (40). Nevertheless, we were able to retransform *E. coli* MC1061 with a DNA isolate from *Serratia* species carrying pPLGN1HIL2. Reisolated plasmid DNA showed an identical restriction pattern as the parent transforming vector. No anomalous plasmids were observed during such an analysis. It seems likely, therefore, that pPLGN1 and pPLGN1HIL2 are maintained structurally stable in the genera tested.

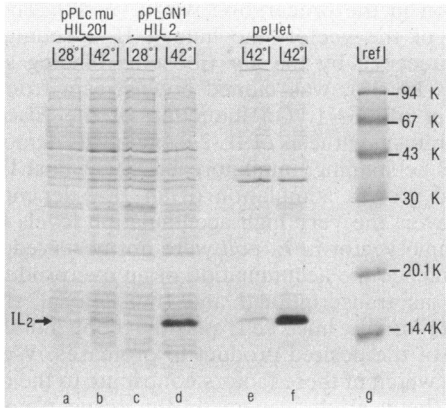

FIG. 2. Polyacrylamide gel electrophoresis of MC1061(pcI857, pPLcMuHIL201) and MC1061(pPLGN1HIL2). Cells were grown at 28°C in LB medium to a density of 5×10^8 cells per ml. A part of each culture was then shifted, and either total cells or pellet fractions were analyzed as described in the text. Lanes a and c represent cultures grown at 28°C; the other lanes represent cultures induced at 42°C. Lane g, protein markers (in thousands; low M_r electrophoresis calibration kit; Pharmacia, Uppsala, Sweden). The protein bands were visualized by using Serva Blue R. The arrow points to the mature human IL-2 band (M_r 15,000).

(ii) Detection of expressed protein. As 42°C is lethal to *Erwinia* species (25), and is at the limit of tolerance for *Serratia* species (18), the usefulness of p_L vectors might be questionable. Therefore, we first tested the protein-synthesizing capacity of the acceptor strains, measuring overall [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material. These results indicated that, at least during the first 60 min of induction, protein synthesis at 42°C is essentially comparable to that at 28°C (data not shown).

Expression studies with pPLGN1HIL2 were then carried out in the same way as described above for *E. coli*. *Erwinia* and *Serratia* species carrying pPLGN1HIL2 were induced, and bacterial samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein with an apparent molecular weight of 15,000 was induced at 42°C (Fig. 3). This band was absent in the plasmidless control cultures. In the case of *E. carotovora*, it was difficult to visualize an IL-2 band when total cells were analyzed. However, IL-2 synthesis could be demonstrated in pellet fractions by Serva Blue R staining. Immunological activity of the newly introduced protein was tested by blotting the polyacrylamide gel onto a nitrocellulose filter, as described above. The filter was impregnated with IL-2 antiserum, and after careful washing it was incubated with the second alkaline phosphatase-labeled antibody. One major band could be visualized in a lane at 42°C, while it was absent at 28°C (Fig. 4). Furthermore, the biological activity of the product was determined. Induced cells were concentrated 10-fold and were opened by sonication. The supernatant that was obtained after centrifugation was assayed for its ability to stimulate growth of an IL-2-dependent cell line (Table 3). A significant titer, albeit lower than that obtained with *E. coli*, was detected in the induced extracts of both *Serratia* and *Erwinia* species, indicating that biologically active IL-2

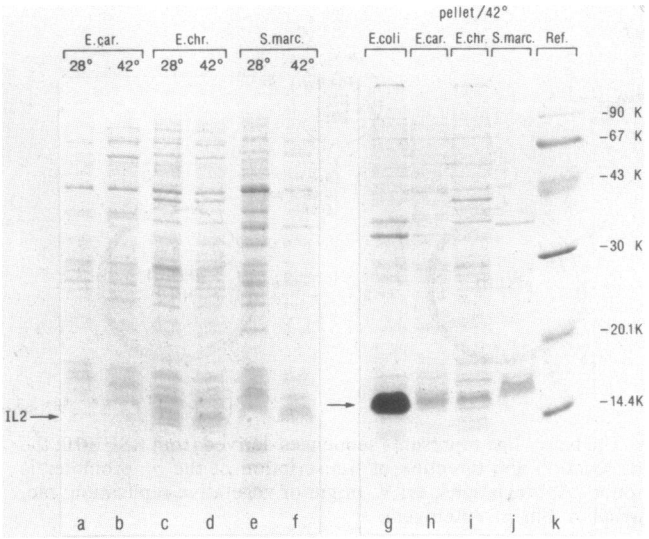

FIG. 3. Polyacrylamide gel electrophoresis of *E. chrysanthemi*, *E. carotovora*, subsp. *carotovora*, *S. marcescens*, and *E. coli*, all of which carried pPLGN1HIL2. Cells were grown at 28°C to a density of 5×10^8 cells per ml. A part of each culture was then shifted to 42°C and incubated for another 2-h period. The cells were then concentrated 10-fold and opened by sonication. Cell debris was spun down, suspended in sample buffer, and boiled for 5 min. Pellet fractions and total cells were analyzed on 15% polyacrylamide gels. Molecular weight markers are indicated (in thousands) to the right.

was synthesized under control of the p_L promoter. No IL-2 activity was detected at 28°C.

In a subsequent experiment we determined the cellular localization of the induced protein. Induced cells were opened by sonication. The insoluble matter was collected by centrifugation and dissolved by boiling in sample buffer. When analyzed by gel electrophoresis, the bulk of the IL-2 protein was present in the pellet fraction (Fig. 3). We conclude that, as in *E. coli* (12), most of the IL-2 synthesized by *Erwinia* and *Serratia* species is present in an insoluble form.

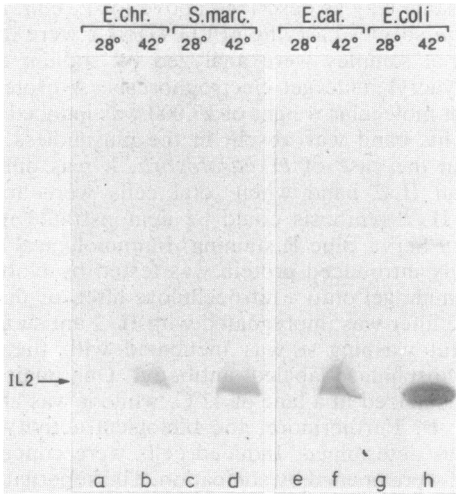

FIG. 4. Western blot analysis of *E. chrysanthemi*, *E. carotovora* subsp. *carotovora*, *S. marcescens*, and *E. coli*, all of which carried pPLGN1HIL2. Pellet fractions were blotted onto a nitrocellulose filter and thereupon characterized with IL2 antiserum, as described in the text.

TABLE 3. Expression of IL-2 by plasmid pPLGN1HIL2[a]

Strain	Temp (°C)	IL2 (U/ml)[b]
Escherichia coli MC1061	28	<1
	42	4,200
Erwinia chrysanthemi	28	<1
	42	600
Erwinia carotovora	28	<1
	42	525
Serratia marcescens	28	<1
	42	780

[a] Cultures of 10 ml were grown at 28°C and induced for 2 h at 42°C. Cells were concentrated 10-fold and lysed by sonication as described in the text. Samples were serially diluted and tested for their ability to stimulate growth of an IL-2 dependent cell line.

[b] IL-2 units were calculated as described previously (17).

DISCUSSION

In this report we have described the cloning of the strong leftward promoter of coliphage λ onto a derivative of the broad-host-range plasmid RSF1010 (19) and its application for efficient synthesis of cloned gene products in *E. coli* and other enteric bacteria, namely the genera *Serratia* and *Erwinia*. The newly constructed vector pPLGN1 contains the p_L promoter together with the *c*I857 repressor allele, which are cloned into pKT240 (3).

pRK2073 (26) was used to mobilize pPLGN1 and its derivatives to *S. marcescens*, *E. chrysanthemi*, and *E. carotovora* subsp. *carotovora*. Transformation of these strains was unsuccessful, presumably due to the presence of a functional restriction-modification system that may interfere with the propagation of foreign DNA, as has been shown for *Pseudomonas* species (1, 20). In addition, *Serratia* species secrete DNA-hydrolyzing enzymes into the medium (18), forming an efficient barrier against the uptake of naked plasmid DNA. It seems conceivable that obstacles of this kind might be circumvented by introducing heterologous DNA through conjugation, which is indeed a widespread phenomenon in the procaryotic world (9, 42). To test the usefulness of the vector, the human IL-2 coding region, which is preceded by the *ner* ribosome binding site from phage Mu (10, 12), was cloned downstream from the p_L promoter in pPLGN1. On induction of the *Erwinia* and *Serratia* strains, synthesis of IL-2 protein was demonstrated by Serva Blue staining, immunoreactivity against IL-2 antiserum, and growth stimulation of an IL-2-dependent cell line. However, the very high accumulation levels obtained with the same vector in *E. coli* were not observed. Several factors influence the accumulation of an overproduced protein, such as transcriptional and translational efficiency, growth rate of the host cell, plasmid copy number, and sensitivity of the desired product to proteases. We did not investigate which of these factors contribute to the observed differences between the genera. In this respect, notice that relative promoter strength seems to differ even among different *E. coli* strains (29). Because several strains of *Erwinia* are known to be sensitive to the mutator phage Mu (15), it is perhaps not surprising that the *ner* ribosome binding site that is used to direct the synthesis of IL-2 is functional in this strain. Using two very sensitive detection techniques for IL-2 (immunoblotting and a specific biological assay), we were able to show that repression of the p_L promoter is virtually complete at 28°C. These results indicate that the

genera *Erwinia* and *Serratia* share with *E. coli* the property of recognizing the *c*I messenger as translationally competent, despite the fact that it is devoid of a Shine-Dalgarno sequence (30) and can provide sufficient repressor molecules to completely shut off transcription from the p_L promoter at 28°C. We conclude that the activity of the p_L promoter is regulated in these genera as tightly as it is in *E. coli*. This property may be particularly advantageous for the cloning of genes whose products might be toxic to the new host.

When overproduced in *E. coli* many foreign proteins appear to be largely insoluble (12, 36). We found that IL-2 synthesized in *Erwinia* and *Serratia* species is likewise associated with the pellet fraction following sonication and centrifugation.

Recently, Murooka and Mitani (29) demonstrated that λ-promoter sequences are recognized in *Serratia*, *Citrobacter*, and *Klebsiella* species. To these we hereby add *E. chrysanthemi* and *E. carotovora*. Murooka and Mitani (29) used a vector based on a ColE1-derived replicon that has a limited host range. The vector presented here is derived from an RSF1010 replicon and should have retained the property of replicating in a wide variety of gram-negative hosts, including *Pseudomonas*, *Acetobacter*, *Rhizobium*, *Agrobacterium*, and other species (2).

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