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Characterization of Transposon Insertion Out⁻ Mutants of *Erwinia carotovora* subsp. *carotovora* Defective in Enzyme Export and of a DNA Segment That Complements *out* Mutations in *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi*†

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Soft-rotting *Erwinia* spp. export degradative enzymes to the cell exterior (Out⁺), a process contributing to their ability to macerate plant tissues. Transposon (Tn*5*, Tn*10*, Tn*10-lacZ*) insertion Out⁻ mutants were obtained in *Erwinia carotovora* subsp. *carotovora* 71 by using plasmid and bacteriophage lambda delivery systems. In these mutants, pectate lyases, polygalacturonase, and cellulase, which are normally excreted into the growth medium, accumulated in the periplasm. However, localization of the extracellular protease was not affected. The Out⁻ mutants were impaired in their ability to macerate potato tuber tissue. Out⁺ clones were identified in a cosmid library of *E. carotovora* subsp. *carotovora* 71 by their ability to complement mutants. Localization of cyclic phosphodiesterase in the periplasm indicated that the Out⁺ plasmids did not cause lysis or a nonspecific protein release. The Out⁺ derivatives of the *E. carotovora* subsp. *carotovora* 71 mutants regained the ability to macerate potato tuber tissue. Our data indicate that a cluster of several genes is required for the Out⁺ phenotype. While one plasmid, pAKC260, restored the Out⁺ phenotype in each of the 31 mutants of *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi*, it failed to render *Escherichia coli* export proficient. Homologs of *E. carotovora* subsp. *carotovora* 71 *out* DNA were detected by Southern hybridizations in subspecies of *E. carotovora* under high-stringency conditions. In contrast, *E. chrysanthemi* sequences bearing homology to the *E. carotovora* subsp. *carotovora* 71 *out* DNA were detectable only under low-stringency hybridization. Thus, although the *out* genes are functional in these two soft-rotting bacterial groups, the genes appear to have diverged.

A number of gram-negative bacterial species belonging to the genera *Erwinia* and *Pseudomonas* cause tissue-macerating or soft-rot diseases in a wide variety of plants (26, 27, 33). A common determinant of these diseases is the production of extracellular degradative enzymes by the pathogenic bacteria. Examples of such enzymes are pectate lyase (Pel) and pectin lyase, polygalacturonase (Peh), pectin methyl esterase, cellulase (Cel), protease (Prt), and phospholipase (12, 14). While the specific contribution of each of those enzymes in the elicitation of soft-rot diseases awaits clarification, both genetic and physiological evidence indicates an important role of Pels (3, 5, 32, 42). Moreover, studies with mutants of *Erwinia carotovora* subsp. *carotovora* (4, 8), *Erwinia chrysanthemi* (1, 43), and *Pseudomonas viridiflava* (27) have demonstrated that enzyme export is tightly linked with the ability of these bacteria to cause the soft-rot disease.

Enzyme export is a trait found in many gram-negative bacteria in addition to the soft-rotting *Erwinia* and *Pseudomonas* spp. (21, 34, 36). In fact, mutants of the plant pathogens *Pseudomonas solanacearum* (38) and *Xanthomonas campestris* pv. *campestris* (16) defective in extracellular Peh and Pel, respectively, were less virulent. These findings illustrate the generalized significance of protein export in bacterial plant pathogenicity. Despite the importance of extracellular proteins in bacterial pathogenicity and biopolymer degradation (12, 34), our understanding of the mechanisms underlying protein export is rather incomplete.

The isolation of nonconditional export-defective (Out⁻) mutants both in *E. chrysanthemi* (1, 22, 43) and in *E. carotovora* subsp. *carotovora* (8, 19) has opened the way to genetic and biochemical analyses required for an understanding of the structure and function of the Out proteins. In *E. chrysanthemi*, *out* genes are scattered on the chromosome (43, 45). Thus far, only one gene, *outJ*, has been cloned from an *E. chrysanthemi* strain (23). Preliminary evidence suggested that the *out* genes of *E. carotovora* subsp. *carotovora* are clustered (8), which raised the possibility of simultaneously cloning most or all of the *out* genes from this bacterium.

In this report, we (i) describe the isolation and characterization of Out⁻ transposon insertion mutants of *E. carotovora* subsp. *carotovora* 71, (ii) report cloning of *E. carotovora* subsp. *carotovora* 71 DNA segments that restored the Out phenotype, (iii) provide evidence for the clustering of the *out* genes, (iv) demonstrate a reconstitution of the Out machinery by cloned DNA in the soft-rotting *Erwinia* species but not in *Escherichia coli*, and (v) report the occurrence of sequences homologous to the *E. carotovora* subsp.

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carotovora 71 *out* DNA in the subspecies of *E. carotovora* and in *E. chrysanthemi*.

(A preliminary account of some of this work has been published [31].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Bacterial strains, phage, and plasmids are described in Table 1. The strains carrying drug markers were maintained on L agar medium supplemented with the desired antibiotics. The wild-type *Erwinia* strains were maintained on yeast extract-glucose-calcium carbonate (YDC) agar (6). Lambda lysates were kept in SM buffer (28).

Media. L medium, minimal salts medium, polygalacturonate-yeast extract (PY) agar, and YDC agar were described previously (6, 9). Phenol red was omitted when PY liquid medium was used. Pectate-yeast extract-citrus pectin (PYCP) medium was the same as PY medium but was supplemented with 0.1% citrus pectin. When desired, antibiotics were added as follows (micrograms per milliliter): ampicillin, 50; chloramphenicol, 10; gentamicin, 10; kanamycin, 50; spectinomycin, 50; streptomycin, 100; and tetracycline, 10. The minimal medium was supplemented with 50 μg of amino acids per ml as necessary. Media were solidified by the addition of Difco agar (1.5%). Soft (top) agar contained 0.6% Difco agar.

Transposon mutagenesis. Tn*5* insertion mutants were isolated by using the plasmid pJB4JI as previously described (51). Tn*10* and Tn*10-lacZ* mutations were introduced into a Lac$^-$ *E. carotovora* subsp. *carotovora* 71 derivative, AC5006, that was rendered lambda receptive by the introduction of the *lamB*$^+$ plasmid (pHCP2) by the method of Salmond et al. (37). The AC5006 (*lamB*$^+$) construct was infected with an appropriate lambda derivative, and drug-resistant transductants were obtained by the procedure of Way et al. (47).

EMS mutagenesis. For ethyl methanesulfonate (EMS) mutagenesis, the procedure described by Miller (30) was used. Bacterial strains were incubated with EMS for a period that yielded <5% cell survival.

Screening for Out phenotype. Colonies were screened for extracellular pectolytic activity by the PYCP agar assay (10). Extracellular Prt and Cel activities were determined by published procedures (2, 8). Enzymes localized in the periplasm were detected by exposing the colonies to chloroform vapors for 30 min and then incubating them for an additional 4 to 6 h at 28°C before scoring for the enzymatic activities.

Preparation of culture supernatants and cellular fractions for enzymatic assays. After 12 h of growth in appropriate media, the cells were collected by centrifugation (12,000 × *g*; 10 min at 4°C). Periplasmic fractions were obtained by the procedure of Witholt et al. (50), except that a higher EDTA concentration (i.e., 0.25 mM instead of 0.1 mM) was used to increase the efficiency of spheroplast formation in *E. carotovora* subsp. *carotovora* 71 and its derivatives. Cellular extracts were obtained by sonication of the spheroplasts (43). Supernatants, periplasmic fractions, and cellular extracts were assayed for Pel, Peh, and Prt activities without any further treatment. These samples were dialyzed against Tris hydrochloride (10 mM, pH 7.0) before assaying for Cel activity. The procedures for the quantitative assays of Cel, cyclic phosphodiesterase (Cpd), Pel, Peh, and Prt activities and the definitions of the enzymatic units have been described previously (43). Protein content was determined by using BCA reagent (Pierce Chemical Co., Rockford, Ill.).

Isolation of Out$^+$ cosmids. A gene bank of *E. carotovora* subsp. *carotovora* 71 was previously prepared by using the cosmid pSF6 (29, 39). Individual cosmids were transferred by using pRK2013 as the mobilizing system (17) into three randomly selected Out$^-$ mutants of *E. carotovora* subsp. *carotovora* 71. Equal volumes of log-phase cultures of the helper and recipient strains were mixed in 2 volumes of L broth. Samples of 200 μl were added to microtiter wells containing 20 μl of overnight-grown donor cells. Samples of the mixtures (ca. 10 μl) were placed on an L agar surface with a multi-inoculator and incubated at 30°C for 18 to 24 h. The drug-resistant transconjugants were selected on minimal agar containing streptomycin and spectinomycin, grown for another cycle on the same medium, and then replicated on PYCP agar. After 12 h of incubation at 28°C, the colonies were scored for extracellular pectolytic activity. Those with a halo around the colony were considered export proficient.

Recombinant DNA techniques. Standard published procedures were used in the isolation of plasmid and chromosomal DNA, transformation of *E. coli*, restriction digests, gel electrophoresis, electroelution of DNA fragments, DNA ligations in liquid or in soft agarose, and Southern hybridizations (15, 28, 48). After hybridizations, high-stringency washes of the blots were conducted at 63°C: two times for 15 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 30 min in 2× SSC–0.1% sodium dodecyl sulfate, and then 10 min in 0.1× SSC. Low-stringency washes were conducted as described above except that 0.5× SSC–0.1% sodium dodecyl sulfate was used in place of 0.1× SSC. The blots were autoradiographed with intensifying screens and Kodak X-omat RP X-ray film. DNA-modifying enzymes were obtained from Promega Biotec (Madison, Wis.).

Plant tissue maceration. Potato tubers obtained from a local supermarket were soaked in 10% Chlorox for 10 min, rinsed with tap water for 20 min, and air dried at room temperature. Bacteria from an overnight culture on L agar containing appropriate antibiotics were suspended in 55 mM phosphate buffer (pH 7.0). The cell suspensions, adjusted to 200 Klett units, were further concentrated fivefold by centrifugation at room temperature. Potato tubers were punctured, and 10 μl of the suspensions was placed in the wounds. Inoculation sites were sealed with Vaseline, and tubers were placed in a moist chamber at 25°C for 48 h. The extent of tissue maceration was estimated by determining the wet weight of the macerated tissue.

RESULTS

Extracellular enzyme production in *E. carotovora* subsp. *carotovora* 71. *E. carotovora* subsp. *carotovora* 71 secreted at least four degradative enzymes, Pel, Peh, Cel, and Prt, to the culture supernatant during late logarithmic growth (Table 2). However, 15% of the Pel activity and 27% of the Peh activity remained cell bound (Table 2). The concomitant release upon spheroplast formation of cyclic phosphodiesterase and the cell-bound Pel and Peh activities indicated their periplasmic localization.

Pel activity in minimal succinate medium increased about 2- to 3-fold in the presence of pectate and 10-fold in the presence of citrus pectin (data not shown). Since pectin stimulated Pel production and had no apparent adverse effect on Peh and Cel production, media supplemented with pectin were routinely used for the production of these extracellular enzymes.

Isolation of transposon insertion mutants. Two different

TABLE 1. Bacterial strains and plasmids

Bacteria, phage, and plasmids	Relevant characteristics[a]	Reference or source
Erwinia carotovora subsp. *carotovora*		
71	Wild type	51
193	Wild type	51
AH2	Wild type	A. K. Handa
AC3012	*pur* (Ino$^-$) *out* Nal^r	8
AC3015	*qua out* Nal^r	8
AC5006	Lac$^-$	*E. carotovora* subsp. *carotovora* Derived from 71 by EMS mutagenesis
AC5008, AC5009, AC5010, AC5011	*out*::Tn*5* (Km^r)	*E. carotovora* subsp. *carotovora* Derived from 71 (this study)
AC5013, AC5014, AC5015, AC5016	*out*::Tn*10* (Tc^r)	Derived from AC5006 (this study)
AC5017, AC5018, AC5019, AC5020, AC5021	*out*::Tn*10-lacZ* (Tc^r)	Derived from AC5006 (this study)
AH174, AH531, AH549, AH755, AH799, AH1509, AH1694, AH2064	*out*::Mu dl(Ap^r *lac*)	Derived from AH2 (18)
Erwinia chrysanthemi		
EC16	Wild type	7
EC183	Wild type	44
AC4198, AC4199, AC4200	*out*::Tn*5* (Km^r)	43
AC4201	*his-1 thr-1 trp-2* Out$^-$ Str^r	Derived from AC4023 (10) by EMS mutagenesis
AC4205, AC4206, AC4207	*out*::Tn*5* (Km^r) Nal^r	Derived from AC4150 (43) laboratory collection
Erwinia carotovora subsp. *atroseptica*		
12	Wild type	51
AC9007	Out$^-$	*E. carotovora* subsp. *atroseptica* Derived from 12 by EMS mutagenesis (this study)
Escherichia coli		
AC8001	*arg his lac* Str^r Km^r Gm^r (pJB4JI)	11
DW75	*lamB*$^+$ Ap^r Cm^r Tc^r	37
HB101	*gal lacY leu pro recA* Str^r	51
Bacteriophage		
λ840	λ-Tn*10* (Tc^r)	47
λ1045	λ-Tn*10-lacZ* (Tc^r)	47
Plasmids		
pDSK519	Mob$^+$ Km^r	25
pHCP2	*lamB*$^+$ Mob$^+$ Ap^r	13
pJB4JI	Mob$^+$ Tra$^+$ Gm^r Km^r (pPHIJI::Mu::Tn*5*)	20
pLG339	Km^r Tc^r	40
pPHIJI	Mob$^+$ Tra$^+$ Gm^r	20
pPL74	*pelA*$^+$ *pelE*$^+$ Cm^r Tc^r	24
pRK2013	Mob$^+$ Tra$^+$ Km^r	17
pSF6	Mob$^+$ Spc^r Str^r	39
pAKC213	*peh1*$^+$ *pel3*$^+$ Ap^r Tc^r	49
pAKC227	*pel1*$^+$ Ap^r	49
pAKC260, pAKC261, pAKC262, pAKC263	Out$^+$ Spc^r Str^r	pSF6 containing *E. carotovora* subsp. *carotovora* 71 genomic DNA (this study)
pAKC265, pAKC290, pAKC291	Out$^+$ Spc^r Str^r	Deletion derivatives of pAKC260 (this study) (Fig. 2)
pAKC269, pAKC292	Out$^-$ Spc^r Str^r	Deletion derivatives of pAKC260 (this study) (Fig. 2)
pAKC600	Out$^+$ Km^r	Subcloning of *out*$^+$ DNA into pLG339 (this study) (Fig. 2)
pAKC601	Out$^+$ Km^r	Subcloning of *out*$^+$ DNA into pDSK519 (this study) (Fig. 2)

[a] Abbreviations: Nal^r, nalidixic acid resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Str^r, streptomycin resistant; Gm^r, gentamicin resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Spc^r, spectinomycin resistant.

TABLE 2. Levels and localization of extracellular enzymes in *E. carotovora* subsp. *carotovora* 71 and its transposon insertion mutants[a]

Bacterial strain	Transposon insertion	Pel		Peh		Cel		Prt	
		Total units[b]	% of total in S[c]	Total units	% of total in S	Total units	% of total in S	Total units	% of total in S
71	None	57.3	85	81.1	73	5.3	95	9.6	95
AC5006	None	40.7	83	86.5	76	6.0	95	9.3	95
AC5008	Tn*5*	161.6	2	88.5	ND[d]	14.5	ND	14.3	96
AC5013	Tn*10*	63.3	6	81.5	ND	7.6	ND	12.2	96
AC5021	Tn*10-lacZ*	59.9	2	91.1	ND	6.7	ND	8.9	93

[a] Bacterial strains were grown in PYCP medium for about 12 h at 30°C to a Klett value of about 200. Supernatants, periplasmic fractions, and cellular extracts were prepared and assayed for enzymatic activities (8, 43).
[b] Total units = enzymatic activity in cells and supernatant expressed as units of activity per milligram of protein.
[c] S, Supernatant.
[d] ND, No detectable activity (<0.005 U for Peh and Cel).

delivery systems were utilized. For Tn*5* mutagenesis, we used the transmissible plasmid pJB4JI. By mating AC8001 with *E. carotovora* subsp. *carotovora* 71, we obtained a high frequency of Km^r Gm^s colonies, as previously observed (51). Of the 3,000 Km^r Gm^s colonies, 5 were Out^-. For the introduction of Tn*10* and Tn*10-lacZ*, we used the lambda derivatives constructed by Way et al. (47). Upon the introduction of pHCP2, carrying the *E. coli lamB*$^+$ gene (13, 37), several *E. carotovora* subsp. *carotovora* strains became receptive to the lambda particles, although the efficiency of transduction, as indicated by the recovery of Tc^r colonies, varied between strains. With AC5006 carrying pHCP2, we could obtain Tc^r colonies at a frequency of about 10^{-8} to 10^{-7}/PFU. By screening 6,000 Tc^r colonies of AC5006 carrying pHCP2, we obtained 8 Out^- mutants resulting from Tn*10* or Tn*10-lacZ* insertions.

Characterization of mutants. The altered patterns of extracellular protein production and secretion in three *E. carotovora* subsp. *carotovora* 71 Out^- mutants are shown in Table 2. AC5013 and AC5021, typical of the Out^- mutants, produced Pel, Peh, and Cel equivalent to the wild type but failed to export these enzymes. AC5008 is unusual in that it produced about threefold more Pel and Cel but failed to export either of these enzymes or Peh. The secretion of Prt was not affected in all the Out^- mutants analyzed.

The Out^- mutants were impaired in their ability to macerate potato tuber tissue; the extent of maceration with two mutants is shown in Fig. 1. In general, the amount of tissue macerated by the mutants ranged from 0.2 to 0.4 g, whereas with the parent (*E. carotovora* subsp. *carotovora* 71) the amount of macerated tissue consistently exceeded 1.3 g. The presence of pHCP2, i.e., the *E. coli lamB*$^+$ gene, in *E. carotovora* subsp. *carotovora* 71 derivatives had no adverse effect on enzyme export or potato tuber maceration.

Molecular cloning of *out* genes. By mobilizing an *E. carotovora* subsp. *carotovora* 71 DNA library of about 1,000 cosmid clones into AC5008, AC5013, and AC5021, we obtained four cosmids (pAKC260, pAKC261, pAKC262, and pAKC263) that restored the Out^+ phenotype. These four cosmids were then mobilized into additional Out^- mutants of *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi* previously isolated by transpositional and chemical mutagenesis (see Table 1 for the source of the strains). The data (Table 3) revealed four distinct complementation patterns. Since the cosmid pAKC260 restored the Out phenotype in AC5008 (export of Pel, Peh, and Cel) and Pel export in the 30 additional *Erwinia* strains, it was therefore selected for subsequent studies. Out^+ derivatives of the *E. carotovora* subsp. *carotovora* 71 mutants macerated potato tuber tissue as well as *E. carotovora* subsp. *carotovora* 71 (Fig. 1). The amount of tissue macerated by *E. carotovora* subsp. *carotovora* 71 and mutants harboring pAKC260 consistently exceeded 1.3 g.

To determine whether the Out phenotype conferred by the cosmids was due to a specific export or whether it resulted from lysis or "quasi-lysis" as seen with the so-called bacteriocin-release factor (35), we further analyzed AC5008 derivatives for the localization of Pel and Cpd (Table 4). In AC5008(pSF6) (i.e., the strain carrying the vector), 80% of the Pel activity was found in the periplasmic fraction, while 50 to 74% of the activity in the Out^+ derivatives of AC5008

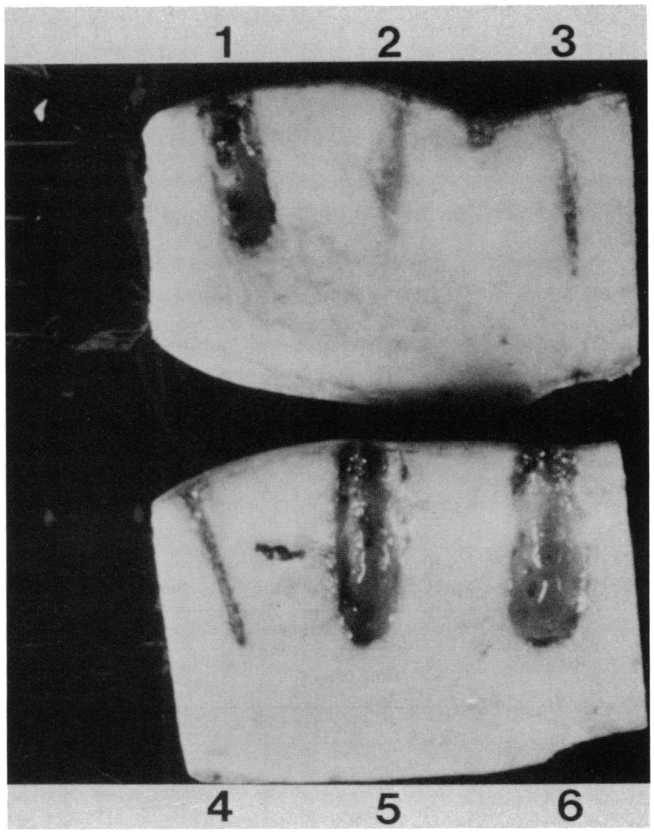

FIG. 1. Potato tuber tissue maceration by *E. carotovora* subsp. *carotovora* (strain 71) and its Out derivatives. Whole potato tubers were inoculated and incubated as detailed in the Materials and Methods. After 48 h of incubation, the tubers were sliced and the extent of maceration was recorded. 1, strain 71(pSF6); 2, AC5008 (pSF6); 3, AC5021(pSF6); 4, buffer control; 5, AC5008(pAKC260); 6, AC5021(pAKC260).

TABLE 3. Restoration of enzyme export in Out$^-$ strains of *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi* by cosmids carrying *E. carotovora* subsp. *carotovora* 71 DNA segments[a]

Bacterial strain[b]	Restoration of Out phenotype with:			
	pAKC260	pAKC261	pAKC262	pAKC263
AC5008	+	+	+	+
AC5011, AC5018	+	+	+	−
AC3012, AC3015, AC5009, AC5010, AC5013, AC5014 AC5015, AC5016, AC5017, AC5019, AC5020, AC5021	+	+	−	−
AC4198, AC4199, AC4200, AC4201, AC4205, AC4206, AC4207, AC9007, AH174, AH531, AH549, AH755, AH799, AH1509, AH1694, AH2064	+	−	−	−

[a] The cosmids were mobilized into the mutants by triparental matings. Transconjugants were selected on glucose-minimal salts agar medium containing streptomycin and spectinomycin. Pectolytic activity was tested on PYCP agar medium by the scoring procedure of Chatterjee and Starr (10).
[b] See Table 1 for the characteristics and source of the Out$^-$ strains.

was exported outside of the cell. In contrast, all the Cpd activity remained cell bound in the Out$^-$ as well as Out$^+$ derivatives of AC5008. The localization of these enzymes in Out$^+$ derivatives of AC5008 (Table 4) and other mutants (data not shown) was similar to that in the wild-type strain, *E. carotovora* subsp. *carotovora* 71. Thus, in the *E. carotovora* subsp. *carotovora* 71 mutants, all the Out$^+$ cosmids mediated a selective export of Pel.

Since pAKC601 (see below and Table 1 for the details of this construct) restored the Out phenotype in every *Erwinia* mutant tested thus far, it was of interest to determine whether the plasmid would render *E. coli* export proficient. For this, we transformed pAKC601 into *E. coli* HB101 carrying the Pel$^+$ plasmids pPL74 and pAKC227 and the Peh$^+$ plasmid pAKC213 (see Table 1 for the characteristics of these plasmids). The plasmid-carrying *E. coli* cells were grown for 14 to 16 h at 30°C in Luria broth containing antibiotics. Culture supernatants, periplasmic fractions, and cellular lysates were assayed for enzymatic activities. The data (Table 5) show that all the Pel, Peh, and Cpd activities remained cell bound in *E. coli* carrying either the Out$^+$ plasmid or the cloning vector pDSK519.

Subcloning of Out DNA from pAKC260. Our first step in the analysis of the *out*$^+$ genes was to reduce the size of pAKC260, which carries ca. 40 kilobases (kb) of *E. carotovora* subsp. *carotovora* 71 DNA. A preliminary analysis of pAKC260 revealed two *Kpn*I sites which were exploited in making pAKC265. This plasmid lacks an *E. carotovora* subsp. *carotovora* 71 DNA segment of ca. 14 kb but retains the ability to restore the Out$^+$ phenotype to all mutants tested. The deletion derivative pAKC269 was obtained by *Hin*dIII restriction endonuclease digest followed by religation (Fig. 2). This plasmid did not confer an Out$^+$ phenotype in any of the four representative mutants (Fig. 2), suggesting that the *out* genes were located on the right-hand side of the pAKC265 insert. This was tested by constructing plasmids carrying the entire 15.7-kb *Sal*I fragment (pAKC600 or pAKC601), the 9.2-kb *Bam*HI-*Sal*I fragment (pAKC290), the 6.5-kb *Sal*I-*Bam*HI fragment (pAKC291), and the 7-kb *Pst*I-*Hin*dIII fragment (pAKC292) (Fig. 2). The *Sal*I fragment was cloned into pLG339 (pAKC600) or pDSK519 (pAKC601), whereas the other fragments were cloned into pSF6. pAKC601 carrying the entire *Sal*I fragment restored the Out$^+$ phenotype in all strains tested and, in this respect, was similar to pAKC265. pAKC290 carrying the 9.2-kb *Bam*HI-*Sal*I fragment complemented AC5008 and AC5018 but not AC5021 or AC9007. In the restoration of the Out phenotype, pAKC290 was similar to the cosmid clone pAKC262. With pAKC291, we noted the restoration of the Out$^+$ phenotype in strain AC9007, derived from *E. carotovora*. subsp. *atroseptica* 12, but not in any of the Out$^-$ mutants derived from the *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains (Fig. 2).

TABLE 4. Localization of Pel and Cpd in *E. carotovora* subsp. *carotovora* AC5008 carrying the Out$^+$ plasmids[a]

Strain and plasmid	Pel		Cpd[b]
	Total units[c]	% of total in S	(total units)
71(pSF6)	61.6	90	3.5
AC5008(pSF6)	173.3	1	3.8
AC5008(pAKC260)	87.5	74	4.1
AC5008(pAKC261)	78.0	59	3.5
AC5008(pAKC262)	78.5	67	4.3
AC5008(pAKC263)	68.3	51	4.3

[a] Cultures were grown for 12 h in PYCP medium. Supernatant (S), periplasmic fraction, and cellular extract were assayed for the enzymatic activities. Cpd was used as a periplasmic marker.
[b] No detectable activity (<0.005 U) in the supernatant.
[c] Total units = enzymatic activities in cellular extract + periplasmic fraction + supernatant expressed as units of activity per milligram of protein.

Occurrence of *out* homologs in *Erwinia* spp. Southern hybridizations were conducted to determine the presence of overlapping *out* sequences in pAKC260, pAKC261, pAKC262, and pAKC263 and of *out* homologs in *E. carotovora*. subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, and *E. chrysanthemi*. The 9.2-kb *Bam*HI-*Sal*I and the 6.5-kb *Sal*I-*Bam*HI fragments from pAKC290 and pAKC291, respectively (Fig. 2), were used as probes against *Sal*I-restricted DNA of pAKC260, pAKC261, pAKC262, and pAKC263. The 9.2-kb fragment hybridized to all these plasmids (Fig. 3A). In contrast, the 6.5-kb *Sal*I-*Bam*HI fragment hybridized strongly to pAKC260 and pAKC261, weakly to pAKC262, and not at all to pAKC263 (Fig. 3B). These data suggest the presence of a truncated version of the 6.5-kb *Sal*I-*Bam*HI fragment in pAKC262 and the absence of these sequences in pAKC263.

The 15.7-kb *Sal*I fragment from pAKC600 was subsequently used as a probe against *Hin*dIII-digested genomic DNA from *E. carotovora* subsp. *carotovora* 71, and 193, *E. carotovora* subsp. *atroseptica* 12, and *E. chrysanthemi* EC16 and EC183 (Fig. 4A). With *E. carotovora* subsp. *carotovora* 71 and 193, strong signals were observed with an 11.0-kb fragment and weaker signals were seen with an 18.3-kb fragment and a 7.4-kb fragment. The 11.0-kb fragment from *E. carotovora* subsp. *carotovora* 71 corresponds to the 11.0-kb internal *Hin*dIII fragment of pAKC601 (Fig.

TABLE 5. Localization of pectolytic enzymes and Cpd in *E. coli* HB101 carrying the Pel$^+$ plasmids and a Peh$^+$ plasmid[a]

HB101 carrying plasmids:	Relevant characteristics	Pel		Peh		Cpd
		Total units[c]	% of total in S	Total units	% of total in S	(total units)[b]
pPL74, pDS519	*pel*$^+$, *out*	0.37	ND[d]	—[e]	—	0.44
pPL74, pAKC601	*pel*$^+$, *out*$^+$	0.43	ND	—	—	0.39
pAKC213, pDSK519	*peh*$^+$, *out*	—	—	1.54	ND	0.39
pAKC213, pAKC601	*peh*$^+$, *out*$^+$	—	—	1.37	ND	0.44
pAKC227, pDSK519	*pel*$^+$, *out*	0.77	ND	—	—	0.36
pAKC227, pAKC601	*pel*$^+$, *out*$^+$	0.77	ND	—	—	0.33

[a] Cultures were grown for 16 h in L broth containing antibiotics. Supernatant (S), periplasmic fraction, and cellular extract were assayed for the enzymatic activities. Cpd was used as a periplasmic marker. pPL74 = pBR329 carrying the *pelA* and the *pelE* genes of *E. chrysanthemi* EC16; pAKC213 = pBR329 carrying the *peh-1* and the *pel-3* of *E. carotovora* subsp. *carotovora* 71; pAKC227 = pBR329 carrying the *pel-1* of strain 71; pDSK519 = vector control; pAKC601 = pDSK519 carrying the 15.7-kb *out*$^+$ DNA of strain (see Fig. 2).

[b] No detectable activity (<0.005 U) in supernatant.

[c] Total units = enzymatic activity in supernatant, periplasmic fraction, and cellular extract per milligram of protein.

[d] ND, No detectable activity (<0.005 U for Pel and Peh).

[e] —, Enzymatic activities were not determined.

2). The other two weaker signals are due to the two fragments corresponding to the *out* DNA flanking the 11.0-kb *Hin*dIII fragment. When the 6.5-kb *Bam*HI-*Sal*I fragment from pAKC291 was used as the probe (Fig. 4B), signals were detected with the 11.0-kb fragment and a 7.6-kb fragment of the *E. carotovora* subsp. *carotovora* 71 strain. Within the 7.6-kb *Hin*dIII fragment, only about 0.5 kb consists of *out* DNA (Fig. 2), accounting for the weaker signal with the *Bam*HI-*Sal*I probe. Homologous fragments were also observed with *E. carotovora* subsp. *atroseptica* 12; while three fragments (7.0, 4.9, and 3.8 kb) hybridized with the 15.7-kb *Sal*I probe, two fragments (6.7 and 3.9 kb) hybridized with the 6.5-kb *Bam*HI-*Sal*I fragment. Hybridization signals following high-stringency washes were not detected when the genomic digests of *E. chrysanthemi* strains were probed with either the 15.7-kb *Sal*I fragment or the 6.5-kb *Bam*HI-*Sal*I fragment. However, under low-stringency conditions (see Materials and Methods for the details), hybridization signals, albeit weak, were detected when the genomic digests of *E. chrysanthemi* were probed with the 15.7-kb *Sal*I fragment (data not shown).

DISCUSSION

E. carotovora and *E. chrysanthemi* are unique among the enterobacteria in their ability to secrete a variety of polysaccharidases and to cause plant diseases involving extensive tissue maceration. Our exploration of the *out* genes in *E. carotovora* subsp. *carotovora* 71 yielded insights into the physiology and genetics of the pathologically important process of protein export in *E. carotovora* and revealed surprising differences between the *out* genes of these two species.

The phenotypes we observed with Out$^-$ mutants of *E. carotovora* subsp. *carotovora* 71 are consistent with those previously observed with *E. chrysanthemi* in indicating that Pel, Peh, and Cel share a common export pathway involving a periplasmic intermediate. In both species, the prevalent export-deficient mutants accumulate Pel, Peh, and Cel in the periplasm, while enzyme synthesis continues at wild-type levels (1, 22, 43). Also, in both species, Prt is exported by a different pathway which is not affected by *out* mutations that block Pel, Peh, and Cel export (Table 2) (also see references 2, 46, and 49).

Our observations with *out* genes cloned from *E. carotovora* subsp. *carotovora* 71, however, indicate a fundamental difference in the arrangement of these genes in *E. carotovora* and *E. chrysanthemi*. Previous work with *E. chrysanthemi* has demonstrated that at least three different chromosomal loci control the Out phenotype (22, 23, 43, 45). The data presented here strongly suggest a clustering of the *out* genes

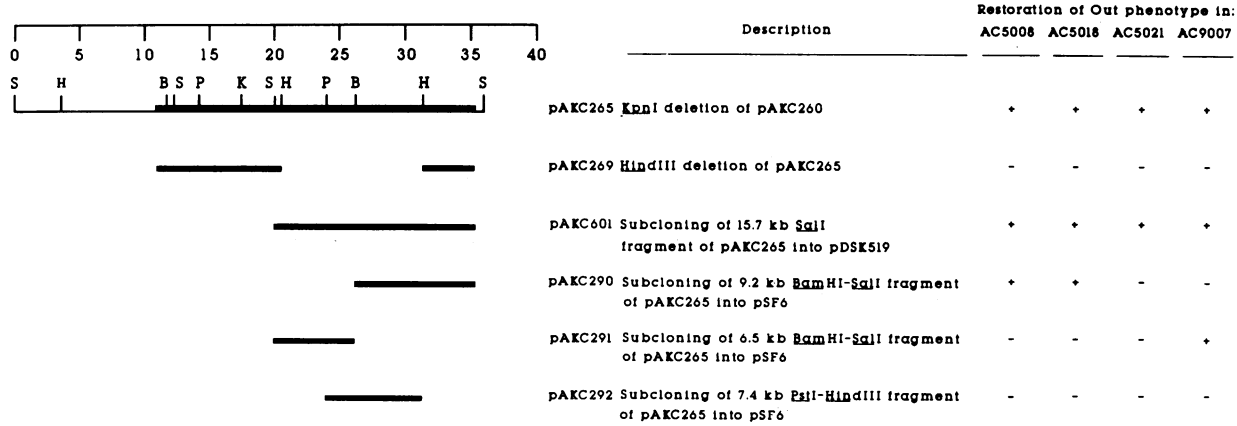

FIG. 2. Analysis of *out* cluster in pAKC265. The bold lines represent *E. carotovora* subsp. *carotovora* 71 genomic DNA, and the thin lines indicate the vector (pSF6) DNA. Restoration of Out phenotype by the plasmids was tested by agar plate assays for pectolytic and cellulolytic activities. See Tables 1 and 3 for the characteristics of the bacterial strains. Abbreviations: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

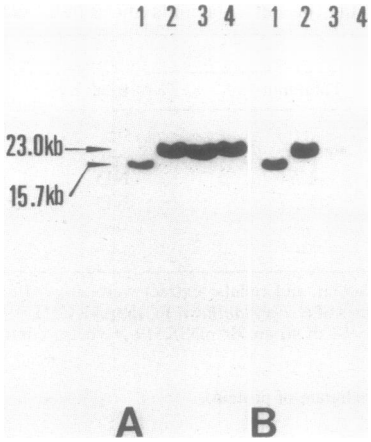

FIG. 3. Southern hybridization of *out*$^+$ plasmids. DNA fragments of *out*$^+$ plasmids generated by the restriction endonuclease *Sal*I were transferred to Biotrans nylon membranes (ICN, East Hills, N.Y.) and probed with *E. carotovora* subsp. *carotovora* 71 *out*$^+$ DNA fragments prepared from gel-purified inserts from the following plasmids. Blot A was probed with the 9.2-kb fragment of pAKC290, and blot B was probed with the 6.5-kb fragment of pAKC291. See Fig. 2 for the *out*$^+$ DNA segments carried by these plasmids. Lanes: 1, pAKC260; 2, pAKC261; 3, pAKC262; 4, pAKC263.

in *E. carotovora* subsp. *carotovora* 71. Cosmids selected on the basis of their ability to complement several *out* mutants contained overlapping fragments. Furthermore, cosmid pAKC260 and its deletion derivatives pAKC265 or pAKC601 restored the Out phenotype to all the transposon-induced mutants of *E. carotovora* subsp. *carotovora* 71 generated in this study and to all the Out$^-$ mutants of other strains of *E. carotovora* and *E. chrysanthemi* (Table 3).

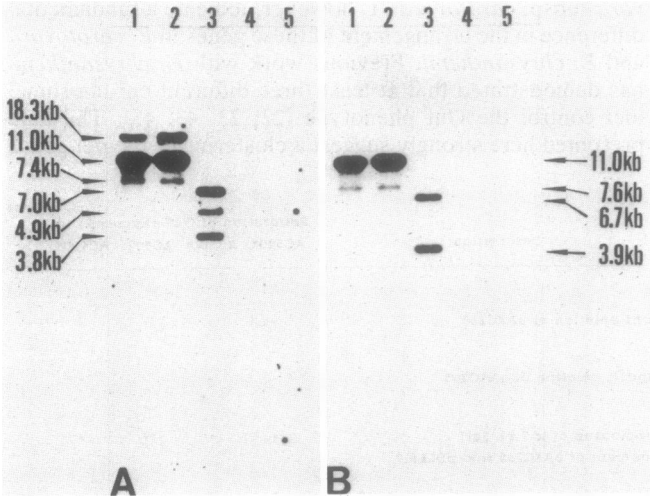

FIG. 4. Southern hybridization of *Hin*dIII-digested chromosomal DNAs from *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi*. The 15.7-kb *Sal*I fragment of pAKC600 (A) and the 6.5-kb fragment of pAKC291 (B) labeled with [^{32}P]dCTP were used as probes. Further details of these plasmids can be found in Fig. 2 and Table 1. Lanes: 1, *E. carotovora* subsp. *carotovora* 71; 2, *E. carotovora* subsp. *carotovora* 193; 3, *E. carotovora* subsp. *atroseptica* 12; 4, *E. chrysanthemi* EC16; 5, *E. chrysanthemi* EC183.

Thus, a functional set of *out* genes appear to be clustered in a 15.7-kb *Sal*I fragment of the *E. carotovora* subsp. *carotovora* 71 genome. Further analysis of this DNA fragment is in progress to define the limits of the *out* genes, determine transcriptional units, and explain differences in the complementation patterns of the plasmids derived from pAKC265 (Fig. 2; Table 1). A clustered arrangement of cloned *out* genes has also been observed by G. Salmond and co-workers (personal communication) with *E. carotovora* subsp. *carotovora* HC131.

Several lines of evidence suggest that the export mediated by the Out$^+$ plasmids is specific. In *E. carotovora* subsp. *carotovora* 71 mutants restored to export proficiency, periplasmic proteins were not released to the medium, while the exoproteins Pel, Peh, and Cel were. Moreover, the *E. carotovora* subsp. *carotovora* mutants complemented with *out*$^+$ DNA were no more sensitive to lysozyme treatment than the parent. Also, the requirement of a gene cluster for the phenotype and the observation of nonoverlapping complementation with several *out*$^+$ plasmids argue against the activity of a lysis protein similar to the bacteriocin-releasing factor (35).

The failure of the clone to enable *E. coli* to export Pel provides further evidence against the action of a nonspecific lysis protein. *E. coli* constructs carrying the *out*$^+$ and *pel*$^+$ plasmids retained Pel activity in the periplasm (Table 5). There are several possible explanations for the failure of the *E. carotovora* subsp. *carotovora* 71 *out* cluster to confer the Out$^+$ phenotype to *E. coli*. The cluster may not contain a full complement of the *out* genes required to reconstitute the entire Out machinery. The proper assembly and localization of the *out* gene products may require other *trans*-acting factors unique to the erwinias. Alternatively, altered regulation of *out* gene expression in *E. coli* may result in the production of nonstoichiometric amounts of the Out proteins, impaired assembly of the export machinery, and aberrant physiological responses. Consistent with this possibility is our failure to clone the *out* genes into high-copy-number cloning vectors and our observation that such recombinant plasmids invariably suffer deletions (data not shown).

Our data indicate homology between the *out* genes of the subspecies of *E. carotovora* as well as *E. chrysanthemi*. However, in *E. chrysanthemi* at least some of the *out* sequences must have diverged since hybridization signals were detected only under low-stringency conditions. Homology between the *out* genes in these soft-rotting bacteria is not unusual since with several other bacteria, homology has been observed in genes encoding accessory (= Out) proteins, for example, those that mediate the export of hemolysin, adenyl cyclase, and leukotoxin (41). Furthermore, our data have shown that the *E. carotovora* Out machinery is able to export the *E. chrysanthemi* Pel. A better understanding of the evolutionary and functional relationship of the protein export systems in *E. carotovora* and *E. chrysanthemi* will develop from further complementation analysis involving additional Out mutants and *out* subclones and from comparison of the sequences of the *out* genes in these two bacteria.

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