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The Three Genes *lipB*, *lipC*, and *lipD* Involved in the Extracellular Secretion of the *Serratia marcescens* Lipase Which Lacks an N-Terminal Signal Peptide

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The extracellular lipase of *Serratia marcescens* Sr41, lacking a typical N-terminal signal sequence, is secreted via a signal peptide-independent pathway. The 20-kb *Sac*I DNA fragment which allowed the extracellular lipase secretion was cloned from *S. marcescens* by selection of a phenotype conferring the extracellular lipase activity on the *Escherichia coli* cells. The subcloned 6.5-kb *Eco*RV fragment was revealed to contain three open reading frames which are composed of 588, 443, and 437 amino acid residues constituting an operon (*lipBCD*). Comparisons of the deduced amino acid sequences of the *lipB*, *lipC*, and *lipD* genes with those of the *Erwinia chrysanthemi* $prtD_{EC}$, $prtE_{EC}$, and $prtF_{EC}$ genes encoding the secretion apparatus of the *E. chrysanthemi* protease showed 55, 46, and 42% identity, respectively. The products of the *lipB* and *lipC* genes were 54 and 45% identical to the *S. marcescens hasD* and *hasE* gene products, respectively, which were secretory components for the *S. marcescens* heme-binding protein and metalloprotease. In the *E. coli* DH5 cells, all three *lipBCD* genes were essential for the extracellular secretion of both *S. marcescens* lipase and metalloprotease proteins, both of which lack an N-terminal signal sequence and are secreted via a signal-independent pathway. Although the function of the *lipD* gene seemed to be analogous to those of the $prtF_{EC}$ and *tolC* genes encoding third secretory components of ABC transporters, the *E. coli* TolC protein, which was functional for the *S. marcescens* Has system, could not replace LipD in the LipB-LipC-LipD transporter reconstituted in *E. coli*. These results indicated that these three proteins are components of the device which allows extracellular secretion of the extracellular proteins of *S. marcescens* and that their style is similar to that of the $PrtDEF_{EC}$ system.

The 62-kDa extracellular lipase of *Serratia marcescens* has no typical N-terminal signal sequence, but a sequence consisting of multiple repeats of nine amino acid residues (GGXGXD XXX), which is characterized as a glycine- and aspartic acid-rich region, is situated in the C-terminal moiety. This sequence was found in the following extracellular proteins of gram-negative bacteria: metalloprotease from *Erwinia chrysanthemi* (5, 6); hemolysin, encoded by the *hlyA* gene in *Escherichia coli* (9); leukotoxin, encoded by the *lktA* gene in *Pasteurella haemolytica* (26); cyclolysin, a multifunctional protein carrying an adenylate cyclase activity and a hemolytic activity, encoded by the *cyaA* gene in *Bordetella pertussis* (11); and Ca^{2+}-binding protein, encoded by the *nodO* gene in *Rhizobium leguminosarum* (7). The colicin V protein, the *cvaC* gene product of *E. coli* (10), possesses a repeated glycine-rich sequence which is not homologous to the GGXGXDXXX sequence but shares some characteristics with it. Since the *E. coli* cells carrying the *S. marcescens lipA* gene encoding the lipase did not secrete the lipase protein into the medium, the lipase is expected to be secreted extracellularly via a signal peptide-independent secretion pathway as described previously (47).

Secretion of the extracellular protein is performed by a specific device composed of three gene products. The genes encoding the secretion device for the extracellular protein lacking the signal sequence have been reported in several bacteria: the *E. coli hlyBC* and *tolC* genes (2, 27, 45, 48) for hemolysin, the $prtDEF_{EC}$ genes (21) for the metalloprotease in *E. chrysanthemi*, the *aprDEF* genes for *Pseudomonas aeruginosa* alkaline protease (12), the *cyaBDE* genes for cyclolysin of *B. pertussis* (10), and the *lktBD* genes for the *P. haemolytica* leukotoxin (42). It has been reported that the cells carrying the *hlyBC* and *tolC* or $prtDEF_{EC}$ genes secrete some of the extracellular proteins described above (3, 8, 22, 28, 39) and that the genes encoding the secretory apparatus for these proteins are similar to the *hlyBC* and *tolC* or $prtDEF_{EC}$ genes. Recently, the *hasD* and *hasE* genes coding for two components HasD and HasE, a secretion device for the *S. marcescens* metalloprotease, have been cloned, respectively (23). The HasD and HasE proteins were highly homologous to $PrtD_{EC}$ and $PrtE_{EC}$, the gene products of the *E. chrysanthemi* $prtDE_{EC}$ genes. The third component of secretion machinery for the *S. marcescens* metalloprotease and the heme-binding protein HasA (24), which corresponds to the TolC protein for the *E. coli* hemolysin or the $prtF_{EC}$ protein for the *E. chrysanthemi* metalloproteases, was not identified in the *has* locus (23, 25). The lipase and metalloprotease proteins of *S. marcescens* have no signal sequence at the N terminus but contain the repeated sequence in the C-terminal moiety (1). The *hasDE* genes involved in the metalloprotease secretion have been cloned (23). However, the pathways for the lipase and its secretion component are still unclear.

Toward a better understanding of the secretion device of *S. marcescens*, this paper deals with molecular cloning and analysis of the genes involved in extracellular *S. marcescens* lipase secretion and extracellular secretion of the lipase and metalloprotease proteins in *E. coli* cells carrying the genes.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli K-12		
DH5	*hsdR17* (r_k^- m_k^+) *thi*	44
JM109	Δ(*lac-proAB*) *hsdR17 thi*	51
PB3	*tolC*	31
S. marcescens Sr41		
8000	Wild type	30
TT392	Nuc^- r^- Ap^s Km^s	43
414	Lip^- Prt^-	This study
Plasmids		
pUC18	Ap^r *lacZ*α	51
pUC19	Ap^r *lacZ*α	51
pHSG298	Km^r *lacZ*α	44
pHSG299	Km^r *lacZ*α	44
pMW119	Ap^r pSC101::*lacZ*α	50
pMW219	Km^r pSC101::*lacZ*α	50
pUT18	pUC18::*Ptac*::*rrnBt1t2*	33, 34
pLIPE121	pUC19::*lipA*	1
pHSGE121	pHSG299::*lipA*	This study
pUTE121	pUT18::*lipA*	This study
pMWE121	pMW119::*lipA*	This study
pMWE122	pMW119::*lipA*	This study
pKHE200	pMW119::*lipA*::*lipBCD*	This study
pMWBCD10	pMW219::*lipBCD*	This study
pKHE65	pMWE122::*lipBCD*	This study
pUTPRTA6	pUT18::*prtA*	This study

[a] Abbreviations used for relevant phenotypes are as follows: Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Muc^-, no production of extracellular nuclease; Lip^- Prt^-, no production of lipase and metalloprotease, complemented by the *lipBCD* genes. For additional details concerning plasmid construction, see the text.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* K-12 JM109 and DH5 were used as hosts for the construction of plasmids. The wild-type strain of *S. marcescens* Sr41, 8000 (26), was used as a DNA donor. *S. marcescens* TT392 (43) deficient in a host restriction enzyme was employed for the modification of plasmid DNA to transform *S. marcescens* cells. LB medium (4) was used as a rich medium. For lipase production, the cells of *S. marcescens* were cultured at 30°C in 500-ml flasks containing 60 ml of the lipase medium (29), which was made of 2% Meast S (Asahi Brewery Co., Ltd., Tokyo, Japan), 1% dextrin, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, Tween 80 (polyoxyethylene sorbitanmonooleate), and 0.1% Colorin 102 (Sanyo Chemical Industries, Kyoto, Japan) (pH 7.0). The modified tributyrin agar plate, which was used to detect the lipase activity of transformants, was described previously (1). The concentration of ampicillin and kanamycin added was 200 μg/ml (each).

General methods. DNA manipulations were carried out according to standard procedures (36). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (19). Transformation of *S. marcescens* was done according to the method described previously (43).

Screening of lipase-secreting transformants of *E. coli*. The tributyrin diffusion agar method (17) was used to find the colonies with extracellular lipase activities. *E. coli* DH5 cells transformed with a DNA library from *S. marcescens* were screened on tributyrin agar plates at 37°C for 24 h for colonies forming a clear halo by lipolysis.

Sequence analysis. After subcloning of the restriction fragments onto pUC18, pUC19, pHSG298, and pHSG299, the nucleotide sequence was determined with a DNA sequencer (Applied Biosystems model 373A). The sequence reaction was done by a dideoxy chain termination procedure (37) with fluorescence-labeled primers according to a protocol of the manufacturer (Applied Biosystems). Nucleotide and amino acid sequence data were analyzed by the computer program GENETYX (Software Development, Tokyo, Japan), the protein sequence database SWISS-PROT, and the protein motif database PROSITE.

PCR, cloning, and construction of the *S. marcescens prtA* plasmid. To amplify the *S. marcescens* metalloprotease gene (*prtA*), the following four synthetic oligonucleotides which are derived from the nucleotide sequence of the *prtA* gene from *Serratia* sp. (32) were used as PCR primers: primer A, 5′-GGGAATTCCC ATGCTGGGTTTTGCTGTGG-3′; primer B, 5′-GGTGATATTGGCGACGT CGGCCC-3′; primer C, 5′-GGGAATTCCCATGCTGGGTTTTGCTGTGG-3′; and primer D, 5′-GGTGATATTGGCGACGTCGGCCC-3′. PCRs were performed with the Gene Amp kit (Perkin-Elmer Cetus Corp., Norwalk, Conn.) as described previously (15). The 1.5- and 1.0-kb DNA fragments containing the *prtA* structure gene and the *prtA* promoter region were amplified by PCRs with primer sets A plus B and C plus D, respectively. The *Pst*I-*Hin*dIII-digested 1.5-kb fragment carrying a part of the *prtA* gene and the *Eco*RI-*Pst*I-digested 1.0-kb fragment were cloned into the *Eco*RI-*Hin*dIII sites of pUT18, generating the *S. marcescens prtA* plasmid pUTPRTA6.

Plasmid construction. The *lipA* plasmids were constructed as follows. The *Sal*I-*Sma*I DNA fragment encoding the *lipA* gene from pLIPE121 (1) was inserted into the *Sal*I and blunt-ended *Pst*I sites of pHSG299, resulting in pHSGE121. The *Eco*RI-*Hin*dIII *lipA* fragment of pHSGE121 was subcloned into the corresponding sites of pMW119 and pUT18, generating pMWE121 and pUTE121, respectively.

The 6.5-kb *Eco*RV fragment containing the *lipBCD* genes was subcloned into the *Sma*I site of pMW219 and pMWE122 lacking the *Sal*I and *Hin*dIII sites of pMWE121, generating pMWBCD10 and pKHE65, respectively. Frameshift mutations of the *lipA*, *lipB*, *lipC*, and *lipD* genes on pKHE65 were introduced by blunting the restriction sites of *Bgl*II, *Apa*I, *Sal*I, and *Hin*dIII, to produce pKHE651, pKHE652, pKHE653, and pKHE654, respectively (Fig. 1). Deletion

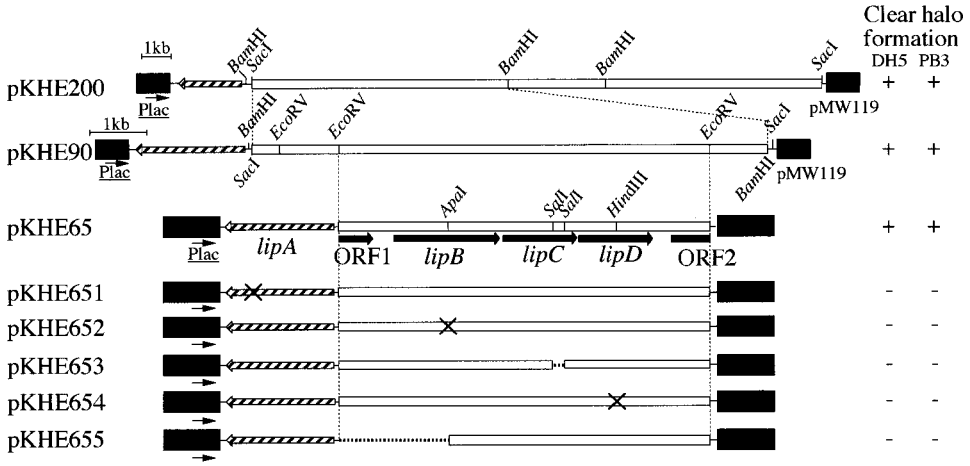

FIG. 1. Restriction endonuclease maps of the inserted DNA of pKHE200 and deletion derivatives and the ability of the recombinant plasmids to give a lipase phenotype to the *E. coli tolC*$^+$ strain DH5 and *tolC* mutant strain PB3. The lipase phenotype was determined by formation of a clear halo on a tributyrin agar plate. The thick arrows indicate the open reading frame determined by sequencing. The cross hatched arrows represent the lipase gene. Open boxes and solid bars represent the chromosomal DNA inserts and vector pMW119, respectively. The *lac* promoter (*Plas*) carried by the plasmid pMW119 is shown by thin arrows. Only the restriction sites used for subsequent experiments are shown. The crosses symbolize the locations of the mutations introduced into the inserted DNA.

mutant plasmids pKHE655 and pKHE656 were created from pKHE65 with the restriction sites *Bam*HI, *Apa*I, and *Sal*I (Fig. 1).

Enzyme assay. Lipase activity of the cultured medium was measured with a Lipase KitS (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) as described previously (18). Units of lipase activity were expressed as micromoles of product formed per minute. Protein concentrations were determined with a Bio-Rad protein assay kit by using bovine serum albumin as the standard protein.

Western-blot (immunoblot) analysis of the gene products. The antiserum against the *S. marcescens* lipase used was described previously (1). Peptide Prt1 (NH_2-HPGDYNAGEGNPTYRDVT-COOH), corresponding to amino acid residues 202 to 219 of *S. marcescens* metalloprotease protein, was synthesized by the *t*-butyloxycarbinyl synthesis strategy. Polyclonal antibody toward the peptide was obtained by injecting rabbits with the peptide in Freund's complete adjuvant as described previously (13). The *E. coli* cells were grown at 37°C in LB medium. The exponentially growing cells were harvested and centrifuged for 10 min at 10,000 × *g* at 4°C. The resultant supernatants were concentrated by precipitation with 10% trichloroacetic acid and then were subjected to SDS-PAGE (12.5% polyacrylamide). The proteins were stained with Coomassie brilliant blue G-250 or electrophoretically transferred to an Immobilon P filter (Millipore) for immunodetection. The blots were blocked by soaking in 5% nonfat dried milk in phosphate-buffered saline and incubated with anti-lipase or anti-metalloprotease antisera (diluted 1:500 to 1:1,000). Signals were detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and the enhanced chemiluminescence (ECL) system (Amersham).

Nucleotide sequence accession number. The *lipBCD* nucleotide sequence data have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D49826.

RESULTS

Cloning of the genes involved in the extracellular secretion of the *S. marcescens* lipase. Although the *E. coli* cells do not possess the secretory pathway for the *S. marcescens* lipase, *E. coli* cells carrying the high-copy-number *lipA* plasmid pLIPE121 accumulated a lot of the lipase protein and showed a lipase secretion phenotype by forming a clear halo on tributyrin plates after 24 h of incubation at 37°C. To clone the secretory genes for the *S. marcescens* lipase in *E. coli*, we constructed the low-copy-number *lipA* plasmid pMWE121 carrying the *lipA* gene in pMW119, which confers a lipase nonsecretion phenotype on cells. The *E. coli* cells harboring pMWE121 cannot form a clear halo on tributyrin plates under the conditions described above (data not shown). Chromosomal DNA from *S. marcescens* 8000, a wild-type strain of Sr41, was digested with *Sac*I, and DNA fragments were ligated to the *Sac*I-digested pMWE121. A DNA library was prepared for the *E. coli tolC*$^+$ strain DH5, and about 50,000 colonies were screened on tributyrin plates. One colony showing clear halo formation was selected as a lipase-secreting clone. The corresponding recombinant plasmid pKHE200, which contained a 20-kb *Sac*I fragment, was isolated (Fig. 1). After subcloning, the gene(s) involved in the extracellular secretion was localized in the 6.5-kb *Eco*RV DNA fragment by using *E. coli* DH5 (Fig. 1). No lipase activity was detected in the cell lysate of the *E. coli* DH5 cells possessing the plasmid pMWBCD10, which encode only the 6.5-kb fragment in the low-copy-number plasmid pMW219. Southern hybridization analysis demonstrated that the cloned *Eco*RV fragment came from the *S. marcescens* chromosome and that there was no rearrangement of DNA. The *lipA* and *prtA* genes were not identified in the 20-kb *Sac*I fragment (data not shown).

Nucleotide sequence of the 6.5-kb DNA fragment and deduced amino acid sequences of the genes. The nucleotide sequence analysis of the 6.5-kb *Eco*RV fragment (Fig. 2) revealed three complete open reading frames (ORFs); one extended from a GTG initiation codon at nucleotide 1054 to a TAA termination codon at nucleotide 2818 and encoded a polypeptide with a size of 588 amino acid residues and with a deduced M_r of 64,173, designated *lipB*. This protein was revealed to possess several highly hydrophobic domains corresponding to transmembrane regions in the N-terminal half and lacks an N-terminal signal sequence. A putative ATP-binding site was found in the central region of the C-terminal half. The sequence AAGGG, which is found 7 bp further upstream, resembles a typical ribosome binding site (40).

The second ORF (*lipC*) starts from an ATG initiation codon at nucleotide 2885 and runs to a TAA termination codon at nucleotide 4204 and encodes a polypeptide with a length of 443 amino acid residues and with a deduced M_r of 49,000. No N-terminal signal sequence was identified in this protein. The last ORF (*lipD*) starts from a TTG initiation codon at nucleotide 4209 and runs to a TGA termination codon at nucleotide 5520 and encodes a polypeptide with a length of 437 amino acid residues and with deduced M_r of 48,311. This polypeptide is rather hydrophilic, and a typical N-terminal signal sequence was found. A putative rho-independent terminator (35) that consists of dyad symmetry following the T-rich clusters is present downstream of the *lipD* gene. These observations indicate that the *lipBCD* genes constitute an operon. A computer search for the promoter sequence (14) revealed the sequence TTGTCA-(20 bp)-TTTTAT, which is located upstream of the *lipB* gene.

Two incomplete ORFs were identified. ORF1 lacking the N-terminal region was located upstream of the *lipBCD* operon in the same direction. This ORF possessed a putative rho-independent terminator structure composed of two dyad symmetries following the T-rich clusters. Downstream of the transcriptional terminator of the *lipBCD* genes, a C-terminal-truncated ORF, ORF2, was observed. The sequences AAGGG and ATG were suggested to be a Shine-Dalgarno sequence and an initiation codon of ORF2, respectively.

Extracellular secretion of the *S. marcescens* lipase in the recombinant *E. coli* cells carrying the *lipBCD* genes. Lipase activity (580 U/ml of the cultured medium) was detected in the cultured medium of *E. coli* DH5 cells harboring pKHE65, which carries the *lipBCD* genes in the low-copy-number *lipA* plasmid pMWE122. No lipase activity was observed in the cultured medium of *E. coli* DH5 cells carrying pMWE122. The *E. coli tolC*-deficient mutant strain PB3 carrying pKHE65 formed a clear halo on tributyrin plates (data not shown), indicating that the *E. coli* TolC protein did not participate in the lipase secretion promoted by the *lipBCD* genes. Western blot analysis revealed that a protein with an M_r of 62,000, which is equal to that of the purified extracellular lipase of *S. marcescens*, was detected in the media of the *E. coli* DH5 cells carrying pKHE65 (Fig. 3). The plasmid pKHE654 encoding the *lipBC* and *lipA* genes could not confer the extracellular lipase phenotype on *E. coli* DH5, showing that the secretion of the lipase by the *lipBCD* genes in *E. coli* was TolC independent. The *E. coli* DH5 cells carrying the *lipBCD* plasmid pMWBCD10 and the high-copy-number *lipA* plasmid pUTE121 showed overproduction of the lipase in the cultured medium. The *S. marcescens* lipase was secreted dependently in response to the *lipA* expression level (Fig. 3).

Extracellular secretion of the metalloprotease via the pathway encoded by the *lipBCD* genes. To know whether the secretion pathway encoded by the *lipBCD* genes is specific for the lipase or not, the extracellular secretion of the *S. marcescens* metalloprotease in the *E. coli* DH5 cells carrying the *lipBCD* genes was examined. The *prtA* plasmid pUTPRTA6 was constructed. The *E. coli* cells possessing both pMWBCD10 and pUTPRTA6 were able to extracellularly secrete the metalloprotease whose size is the same as that of the protein obtained from *S. marcescens* (Fig. 4). The metalloprotease which was not secreted and accumulated inside of the *E. coli* cells was larger than that obtained from *S. marcescens*, indicating that intracellular protein in the *E. coli* cells is not processed. Thus,

FIG. 2. Nucleotide sequence and flanking region of the *S. marcescens lipBCD* genes. The deduced amino acid sequences of the *lipBCD*-encoded proteins are given under the nucleotide sequence. The asterisks indicate a stop codon. The possible ribosome binding sites (Shine-Dalgarno [SD]) are boxed, and putative −10 and −35 sequences are underlined. Potential transcription termination sequences (nucleotides 692 to 718, 729 to 752, and 5619 to 5645) are indicated by arrows.

FIG. 2—*Continued.*

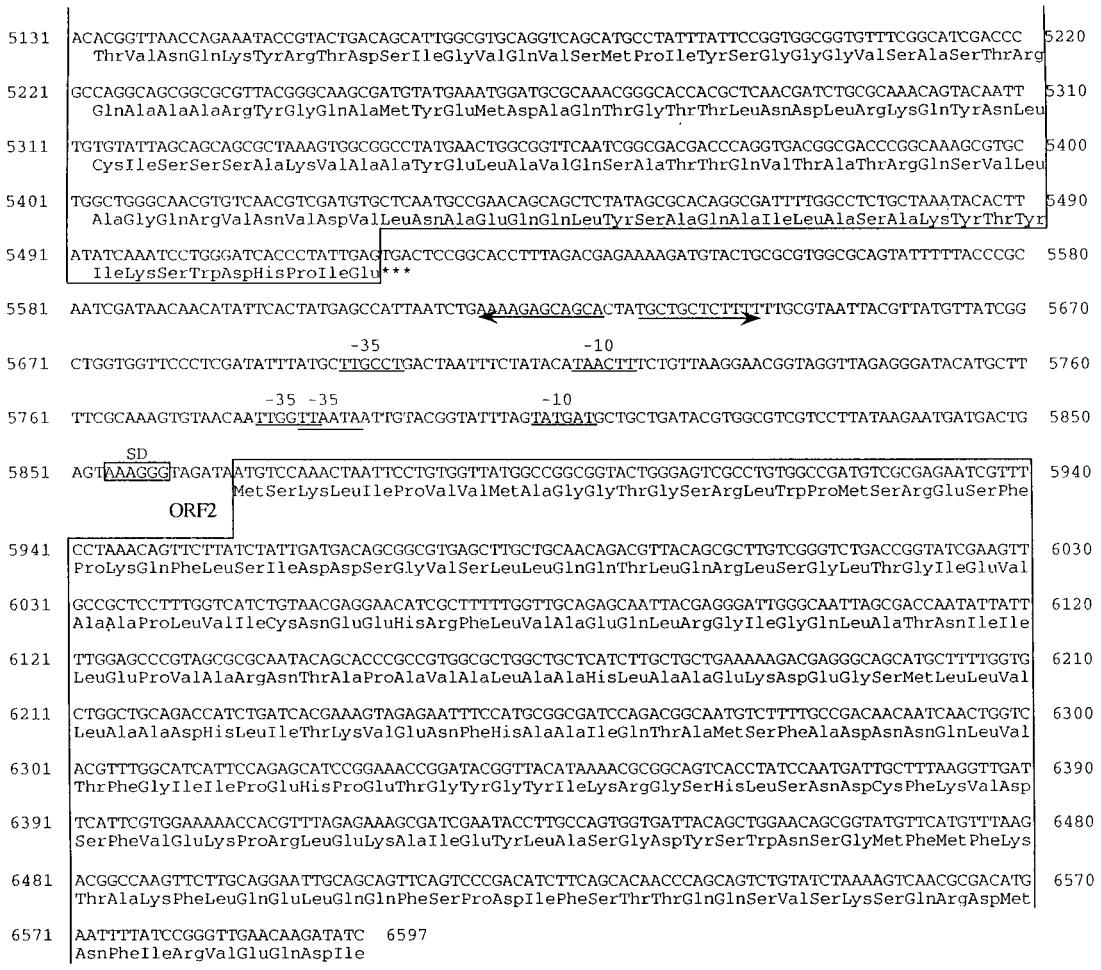

FIG. 2—*Continued.*

it was clearly shown that the LipB, -C, and -D proteins work as the secretion system for both extracellular proteins of *S. marcescens* in *E. coli.*

DISCUSSION

The *S. marcescens* lipase is expected to be secreted into the medium via a signal peptide-independent pathway (1). The *S. marcescens* genes which were cloned by selection of the lipase phenotype with a low-copy-number *lipA* plasmid enabled the extracellular secretion of the *S. marcescens* lipase in *E. coli*. The three genes *lipB*, *lipC*, and *lipD* were identified and constituted an operon.

A search for homology in protein databases revealed that the *lipB* and *lipC* gene products were highly homologous to the products of the *E. chrysanthemi* $prtD_{EC}$ and $prtE_{EC}$ genes (56 and 46% identity) and the *S. marcescens hasD* and *hasE* genes (54 and 45% identity), respectively (Fig. 5 and 6). Two clusters of the $prtDE_{EC}$ (21) and *hasDE* (23, 25) genes encode the ABC transporters of the *E. chrysanthemi* metalloprotease and the *S. marcescens* HasA and metalloprotease, respectively. The sequences GPSASGKS and LSGGQKQRIGLA, which are an ATP- and GTP-binding site motif A (38, 46) and an ABC transporter family signature, respectively, were identified in LipB, indicating the LipB protein belongs to a member of the ABC protein family able to bind ATP. The LipC protein possessed

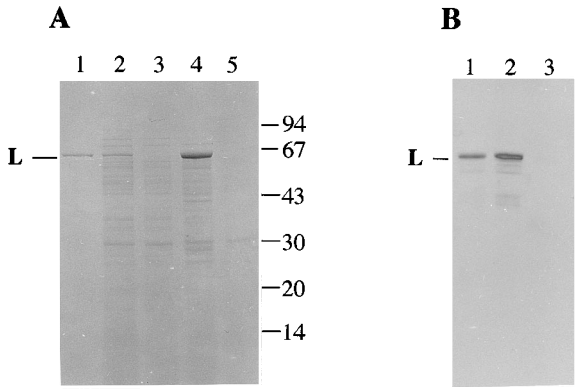

FIG. 3. Analysis of the *S. marcescens* lipase secreted into the culture media of recombinant *E. coli* cells carrying the *lipBCD* genes. The *E. coli* K-12 DH5 cells carrying recombinant plasmids were cultured in LB medium at 37°C for 18 h. The polypeptides in the supernatant of the cultured media were concentrated by trichloroacetic acid precipitation and then subjected to SDS-PAGE (12.5% polyacrylamide) as described in Materials and Methods. The gels were stained with Coomassie brilliant blue G-250 (A) and analyzed by immunoblotting with antiserum against the *S. marcescens* lipase (B). The position of the lipase is shown by L. Optical density (OD) equivalent units of concentrated supernatants of the cultured media are given below in parentheses. (A) Lanes: 1, purified extracellular lipase from *S. marcescens*; 2, pKHE65 (0.5 OD unit); 3, pMWE121 (0.5 OD unit); 4, pMWBCD10 plus pUTE121 (0.1 OD unit); 5, pMW219 plus pUTE121 (0.5 OD unit). (B) Lanes: 1, purified extracellular lipase from *S. marcescens*; 2, pKHE65 (0.5 OD unit); 3, pMWE121 (0.5 OD unit).

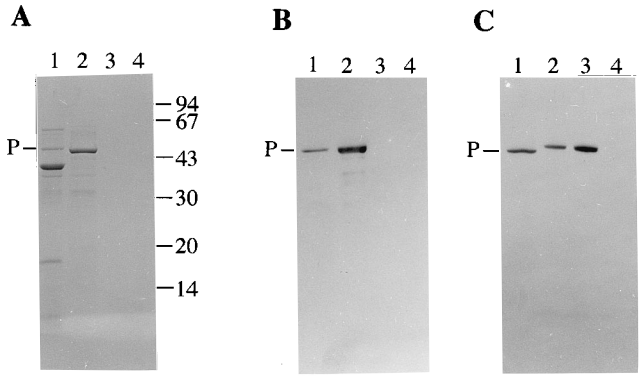

FIG. 4. Analysis of the *S. marcescens* metalloprotease secreted into the culture media of recombinant *E. coli* cells carrying the *lipBCD* genes. The *E. coli* K-12 DH5 cells carrying recombinant plasmids were cultured in LB medium at 37°C for 18 h. The polypeptides in the supernatant of the culture media were concentrated by trichloroacetic acid precipitation. The resultant supernatants (A and B) and cell lysates (C) were subjected to SDS-PAGE (12.5% polyacrylamide) as described in Materials and Methods. The gels were stained with Coomassie brilliant blue G-250 (A) and analyzed by immunoblotting with antiserum against the *S. marcescens* metalloprotease (B and C). The positions of the metalloprotease are shown by P. Optical density (OD) equivalent units of concentrated supernatants of the culture media are given below in parentheses. Lanes: 1, culture medium from *S. marcescens*; 2, pMWBCD10 plus pUTPRTA6 (0.1 OD unit); 3, pMW219 plus pUTPRTA6 (1.0 OD unit); 4, pMWBCD10 plus pUT18 (1.0 OD unit).

the sequence IKPGMPVEVFVKTGSRSLLSYLF, termed the HlyD family secretion protein signature. The products encoded by the *S. marcescens lipB* and *lipC* genes were revealed to be inner membrane components involved in transport across the membrane. The third outer membrane component corresponding to the *E. chrysanthemi prtF*$_{EC}$ gene product was encoded by the *lipD* gene in *S. marcescens* (42% identity) (Fig. 7). The products of the *lipB*, *lipC*, and *lipD* genes showed 26, 20, and 22% identity with the *hlyB*, *hlyD*, and *tolC* gene products of *E. coli*, respectively (data not shown). Thus, the LipB, LipC, and LipD transporters for the extracellular lipase were analogous to transporters PrtD$_{EC}$, PrtE$_{EC}$, and PrtF$_{EC}$ and HasD and HasE (a third component for the Has system has not been identified yet). Deletion mutation analysis indicated that all three *lipBCD* genes were essential for the extracellular secretion of the *S. marcescens* lipase in *E. coli*, and these genes constituted an operon like the *prtDEF*$_{EC}$ genes.

Although the genes involved in the synthesis and secretion of the extracellular proteins are usually clustered, the lipase and metalloprotease genes were not identified in the 20-kb *Sac*I fragment containing the *lipBCD* genes in *S. marcescens*. Two incomplete ORFs were found upstream and downstream of the *lipBCD* operon. ORF1 did not show any homology with the sequences that appeared in the protein databases. ORF2 downstream of the *lipBCD* operon was revealed to be 53, 52, 52, and 52% identical to the N-terminal portions of the *Salmonella typhimurium cpsB* (41), *S. typhimurium rfbM* (16), *E. coli cpsB* (accession no. P24174), and *S. choleraesuis cpsB* (20) gene products, respectively, which were GDP-mannose pyrophosphorylase (EC 2.7.7.22), which is involved in the lipopolysaccharide biosynthesis. Since lipopolysaccharide has been suggested to play an important role in promoting the localization of secretory components such as TolC and PrtF$_{EC}$ in the outer membrane (49), the finding that the genes encoding the lipopolysaccharide biosynthetic pathway were located in contiguity with the *lipBCD* operon is interesting.

The *lipBCD* gene products promoted the extracellular secretion of the *S. marcescens* metalloprotease in *E. coli* DH5 and were revealed to be involved in the secretion of both extracellular proteins lacking the typical N-terminal signal sequence. TolC is functional as a third component in some hybrid ABC transporter systems, and the secretion of the *S. marcescens* metalloprotease in *E. coli* performed by the Has system is TolC dependent (23). However, the *lipD* gene is essential for the secretion of both proteins in the Lip system reconstituted in *E. coli*, and TolC cannot replace LipD. The *prtF*$_{EC}$, *lipD*, *cyaE*, and *aprF* genes encoding a third component of extracellular secretion were situated in the *prtDEF*$_{EC}$, *lipBCD*, *cyaBDE*, and *aprDEF* operons, respectively, and their functions in secretion were unable to be substituted for that of TolC (11, 12, 21). In contrast, the *hlyBD*, *cvaAB*, *lktBD*, and *hasDE* genes were not contiguous to the genes encoding their third secretory components, and TolC protein functions as a third component of the transport systems encoded by these genes (10, 23, 42, 48).

Since the contribution of the *S. marcescens* Has system to lipase secretion has not been reported, we isolated a lipase-nonsecreting mutant, 414, from the wild-type strain of this bacterium to reveal a role of the Lip pathway for the extracellular secretion. The mutant did not secrete either lipase or metalloprotease on either the lipase or LB medium, and the secretion was recovered by the introduction of the *lipBCD* genes (Fig. 8). The *lipBCD* gene products were revealed to constitute a main secretion pathway for both proteins in *S. marcescens* under culture conditions in which the Has system is suppressed. The mutant expressing the Has system, which was

```
SLMIPB  VNQFIPRNEIA-DVIRTRSKVFWTVGIFTAFINLLMLVPSIYMLQVYDRVLPSRNEITLL
SMHASD            M-S.LAAYRRG..GIAL...V......A.AL.............G.RM..A
EWPRTD  ..ASSE.DRSLFG.L.QFRRS..S....S.V..V...A..V...........A.G.G....
                  *        **    * * ** *** *  ********** * *  **

SMLIPB  MLTLIMLGMFGMMSLLEYVRSMVVIRIGSQLDMRLNTRVYTAAYEANLKNGSSDAGQMLS
SMHASD  ....MVV.LYLF.G...W...Q....L.A.M.....Q...D..F.T...T.NPL...A.N
EWPRTD  ....L.A.LCAF.GA..W...LL.V.L.TRI.LA..QD.FN..FAR..EA.DGR..LA.T
        ****   *     *  ** ***  * * *   *  **  *  **   **  *   **  *

SMLIPB  DLTNLRQFLTGSALFAFFDAPWFPIYLLVIFLFNPWLGLFALVG-ALL-LIALAVINEVV
SMHASD  ........A..N............V....V..LH....AL.SA.CDRAGAAG..QSAGCR
EWPRTD  ...L....I..N.......V....LF...L..LH....ML..G.TVVPGGVG..EPASDQ
        *** **** ** ******* ****  *** **  ****  *  *        **

SMLIPB  SKKPLGEASKLSIMSGNLASTNLRNAEVIEALGMLPNLKRRWFGLHQRFLNSQRIASERA
SMHASD  KRLSAEARPGSRCAHTAGQPA......A.A.M...TD.RL..LRQ..Q..LL.NR...KI
EWPRTD  .TAGGSNQQSQQATHLA-D-AQ....D....M...G..R...LAR.Y..ISL.NL.....
                        ****  * * ***  *  **   *  *   *  ***

SMLIPB  SRVTSITKFVRMSLQSLVLGLGGWLAIDGHITPGMMIAGSILMGRTLAPIEQVINVWKSY
SMHASD  AA..AWS.T..LA....M..C.AL..VS.D.............I..V.G..D.L.GA..QW
EWPRTD  AA.GGAS.YS.IA....M....AL.....K.............V..V.S..D.L.G...QW
          *    *  *  **** ** *  **  * ************ ** * ** * *  **

SMLIPB  SAAKLSYGRLVKLLETHPQRGTGMSLPRPEGVLSVEGVTATPPGSKGDAVLHNVSFAIQP
SMHASD  .S.RQ.LQ..EVM.AAN.P.IPSLP..A.G.A.T.SQL..SA..G-TAP...G...RLEA
EWPRTD  .S.RIAWQ..TR.IAAY.P.PAA.A..A...H....Q.SLRTAQG-NTR-.Q.IH.SL.A
        * *      **       * *     ** * * * *                 *    *

SMLIPB  GDVLGIIGPSASGKSTLARLLVGIWPVSEGIVRLDNADIYQWNKDELGPYIGYLPQDIEL
SMHASD  .E...V..A.G...TL.M.Q...ALTPIS.-----.GGA-E.------...H........Q.
EWPRTD  .ET.V.L.A.G....S.......AQSPTQ.K....G..LN.VD.NTF..T.......VQ.
        *  *   * * ***  * * ***          *    *     *       ** *******  *

SMLIPB  FAGTIAENIARFNDIDSEKVIEAAKLAGVHELILRFPNGYDSVIGNGGAGLSGGQKQRIG
SMHASD  ....LTD.....GQV.A...VA..A.....Q...HL.K..ETEL.E..S......R..VA
EWPRTD  .K.SL.......G.A.P...VA.............SL.....TEL.D..G......R....
        * *     *****    * ***  ** ***** ***  * * **     * ** ******* **

SMLIPB  LARALYGDPALVVLDEPNSNLDDAGEKALNQAIMFLKQRNKTVVLITHRTNLLSMTSKLL
SMHASD  .......S..........A...RE..E..QR..EA..A.GN.I..V..KPAI.AT.D...
EWPRTD  ....M....C.LI.....AS..SE.DQ..M...VA.QK.GA........PA.TTLAQ.I.
        **** ** * *  *****  **  *  **  **  *  *  * ** **           * *

SMLIPB  LLVNGNVNAFGPTQQVLQALAN-AQKAQV--PPQAVRAVNSEPDEGEIPKTQIN
SMHASD  V.TA.Q.QH...SDAI.KK.PGF.PA.A.APANTGRSNGGFNVNYANFA..ASGERKV
EWPRTD  I.HE.QQQRM.LARD..TE.Q--QRS.A-NQARM-NPTAAM-.Q
          *  *     *     *  *     *
```

FIG. 5. Comparison of the predicted amino acid sequences of the products of the *S. marcescens lipB*, *S. marcescens hasD*, and *E. chrysanthemi prtD*$_{EC}$ genes. The entire amino acid sequences of the *S. marcescens lipB* (SMLIPB), *S. marcescens hasD* (SMHASD), and *E. chrysanthemi prtD*$_{EC}$ (EWPRTD) genes are shown in one-letter designations. Identical amino acid residues are dotted. Asterisks indicate matches across all of the sequences. The sequences of the putative ATP-binding site and ABC transporter family signature are boxed and underlined, respectively. To maximize homology, gaps (dashes) were introduced into the sequences.

```
SMLIPC      MSTHIGEPQDSYT-EEIPQDERRFTRMG-WLVVGIGLFGFLAWAAFAPLDKGVASP
SMHASE  MSNQSV-IP.DI-.-TL-SRQF-..G.HL.L.G...L-L.FG...L.GLL.......PVS
EWPRTE  MTGMDI.TQD.LNEAAMRDRASR..E.AL.L.W...L-A.FG...L..LL........VQ
                              ** *  * * ***   *  *** *   *******

SMLIPC  GSVTVSGNRKTVQAPASGIIKNIAVRDGDKVKAGEVLVQLSQVQAQAQVDSLRDQYYTTL
SMHASE  ...V.A....A..H.SG.VVSQ.Q.HE..R.R..Q..LLMDT.DSRT.R.A..S.HLSNA
EWPRTE  .N.V......VI.HMQG..VDR.Q.K...R.A..Q..LT.NA.D.RTTSEG.GS..DQLI
        * * * ****  *    *    * *  ** * ** **     *        *  *

SMLIPC  ATEGRLLAERDGLSIVTFSPILDAVKDKPRVAEIIALQTQLFASRRQALQSEIDGYKQSM
SMHASE  .QQA..Q......QAIA.P.L.Q.RREE.E.MSLML..Q...T...A.....LAAIAE.I
EWPRTE  .R.A.....QRNQ.SLAAT.R.TQARQR.EM.A.....ED.LR..Q.S.KL....VRA.I
        *   ** **          * *      *        **  *  **   *  *      *

SMLIPC  DGIRFQL-KGLQDSRGNKQIQLSSLREQMNSMKQLAADGYLPRNRYLEVQRQFAEVNSSI
SMHASE  A.SQAM.EGVRRIL.PA..R.KAM.Q..LSG.RNWP.Q.-MW.ASAVGSEG.-HRISTQA
EWPRTE  ..LETS.GALQKVM-SS..SEQAT.SQ.LQGLRP....N.V...KM..TE.L..Q.SGEL
              *    *           **     *  *         *     *

SMLIPC  DETVGRIGQLQKQLLESQQRIDQRFADYQREVRTQLAQTQMDASEFRNKLQMADFDLGNT
SMHASE  SRIP-D..R.GR.-S.LKL.SPA.--E..K..SS..PRYDEA-D.LD.R.AK.EA...H.
EWPRTE  AQ.S.EV.RTRRDIQQQKL..A..QQE.DK..NSE.SDV.AKLN.VISQREK...N.A.V
              *           *   *   *  **    *         *       *   *

SMLIPC  AITSPVDGTVVGLNIFTQGGVVGAGDHLMDVVPSQATLVVDSRLKVDLFDKVYNGLPVDL
SMHASE  QVKA..A......SV..E...I...QQ..EI...DRG.Q.EA.IP.E.I...QV....E.
EWPRTE  QVRA..A....DMK...E...IAP.QVM..I..EDQP.L..G.IP.EMV...WS....E.
          ** ****    ** ***   *   *  **    * *  *  *   ***  **** *

SMLIPC  MFTAFNQNKTPKIPGTVTLVSADRLVDKANGEPYYQMQVTVSPEGMKMLSGEDIKPGMPV
SMHASE  L.S....ST..RVE.E....G....T.EKS.A...SVRAK..E..LQR.N.LE.R.....
EWPRTE  Q....S.ST..RV......L.......EKD.T...GLRIQ..E..KRS.H.LE.......
         * ** *  **   * ***  **** *   * ***      ** **   * *  * *****

SMLIPC  EVFVKTGSRSLLSYLFKPILDRAHTSLTEE
SMHASE  .G.IR..E..MMN.....LT..L.LA....
EWPRTE  QG..R..E..FIN.....LM..M.LA....
          *  ** **   *****  ** *  ****
```

FIG. 6. Comparison of the predicted amino acid sequences of the products of the *S. marcescens lipC*, *S. marcescens hasE*, and *E. chrysanthemi prtE*$_{EC}$ genes. The entire amino acid sequences of the *S. marcescens lipC* (SMLIPC), *S. marcescens hasE* (SMHASE), and *E. chrysanthemi prtE*$_{EC}$ (EWPRTE) genes are shown in one-letter designations. Identical amino acid residues are dotted. Asterisks indicate matches across all of the sequences. The HlyD family secretion protein signature is boxed. To maximize homology, gaps (dashes) were introduced into the sequences.

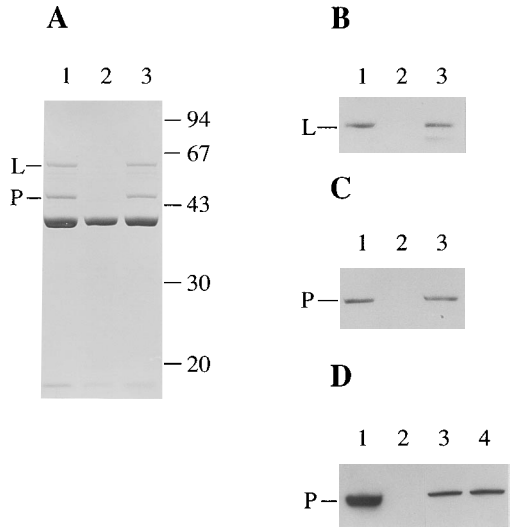

FIG. 8. Extracellular proteins of the *S. marcescens* lipase secretion mutant strain 414. The *S. marcescens* wild-type and mutant cells were cultured in the lipase (A, B, and C), LB (D), and LBD (D) media at 30°C for 18 h. Fifteen microliters of the cultured supernatants was subjected to SDS-PAGE (12.5% polyacrylamide), and the gels were stained with Coomassie brilliant blue G-250 (A) and analyzed by immunoblotting with antiserum for the *S. marcescens* lipase (B) or metalloprotease (C and D). The positions of the lipase and metalloprotease proteins are shown as L and P, respectively. Optical density (OD) equivalent units of concentrated supernatants of the cultured media are given below in parentheses. (A, B, and C) Lanes: 1, *S. marcescens* 8000 (0.2 OD unit); 2, strain 414 (0.2 OD unit); 3, strain 414 carrying pMWBCD10 (0.2 OD unit). (D) Lanes: 1, *S. marcescens* 8000 (LB medium) (0.1 OD unit); 2, strain 414 (LB medium) (0.1 OD unit); 3, *S. marcescens* 8000 (LBD medium) (0.08 OD unit); lane 4, strain 414 (LBD medium) (0.08 OD unit).

cultured on LBD medium (24), secreted the metalloprotease. However the lipase was not detected in the culture supernatant (Fig. 8), showing that the Has system promotes the secretion of the metalloprotease but that the lipase secretion was undetectable. The lipase activities of the wild-type strain 8000 and the lipase-nonsecreting mutant 414 were 44 and <4 U/ml on LB medium and 57 and <4 U/ml on LBD medium, respectively. The third component of the Has system has not been identified. Although a third component in lipase secretion, LipD, was not replaced by TolC in the Lip system constituted in *E. coli*, LipD, like TolC, may function as a third component of the Has system. Reconstruction of the hybrid transport systems in *E. coli* and analysis of the *S. marcescens* mutants deficient in both lipase and metalloprotease secretion will clarify the function of LipD and its cooperation in the Has system.

```
SMLIPD  LIHSKRQAAGLVIGTLLFAMSAPVYSIGILDAYSLALEKDPTFRAAIKEKEAGDENENIG
EWPRTF      M.RK.V.LTVV.SLS-GGSAQAM.L...WE...RN.AQL...GF.RD..Q.EVA..
             *  * *    *           * ***  ***  *   ***  *  ** *   **

SMLIPD  RAGLLPKVSLNYQNSPRNWQTQKYPQSDFFGNVSEVTRRQQYRSYSSSITLTQPLFDYEA
EWPRTF  ......--..Q.TYGANYSHS-.VT.R.--RTLNNT.K.-D.DN.V.TL..R....L..A.
******  ** *          *  * *         * *  *  * *  ** *** ** *

SMLIPD  YARYKAGVAQTMMSDETYRGKLLDLAVRVINAYVEVAYSKDQIALAEAQKAAYKEQLTLN
EWPRTF  W...QQ..TRKL.A.QRF.DRSQ..M..LYQSWS.ALLAQEKLM.LD..RR..Q...A..
         ***  **    * *   *    ** **       *          *  **  ** *** **

SMLIPD  DRLMSAGEGTITDVSETQARYSLAEAQVIEARDALDAAQRELEVIIGMPLNQLDELQVLR
EWPRTF  R..LA.....Q..LR..E...TVT...R..QE.T....MTD..NMM.S..-.IQD.SP.A
         **  ***** **  ** ***   *** **  * ****   **   * ** *   *  *

SMLIPD  PGKFKVAPLIPSKFEEWQKIALENNPVLAASRHGVDAAKYDVERKRAGFMPQVQLYASHS
EWPRTF  LDTLPDNVTENRSLSQ.RELTVRH.AK..VQ.EN..YSR.EI..N...HL.TLD.V..TR
                     *       *  **  *  **   *  ** ***  *   * **

SMLIPD  ENDASSDNTVNQKYRTDSIGVQVSMPIYSGGGVSASTRQAAARYGQAMYEMDAQTGTTLN
EWPRTF  NSLSE.EYNY....D.QTV.L..RV.L....A....M.....E.Q.SQA.L.N..RQ.FA
          *    **** *   * **  * **** **** ***** * *   * * **  *

SMLIPD  DLRKQYNLCISSSAKVAAYELAVQSATTQVTATRQSVLAGQRVNVDVLNAEQQLYSAQAI
EWPRTF  E..R.F...ANGA..IR.WQMS.AA.EEAIR......AG.E.I.L...M...EW.N.RRE
         ** * ***    **  *    *  *      ******  * * * *** ***  * *

SMLIPD  LASAKYTYIKSW-DHPIE
EWPRTF  .TEV..RWLQA.LNLRYTAGTLNEQDMMQLAAWFQSAPVINKTGINAATGNKTN
         *   **    *
```

FIG. 7. Comparison of the predicted amino acid sequences of the products of the *S. marcescens lipD* and *E. chrysanthemi prtF*$_{EC}$ genes. The entire amino acid sequences of the *S. marcescens lipD* (SMLIPD) and *E. chrysanthemi prtF*$_{EC}$ (EWPRTF) genes are shown in one-letter designations. Identical amino acid residues are dotted. Asterisks indicate matches across all of the sequences. To maximize homology, gaps (dashes) were introduced into the sequences.

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