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Lipase Secretion by Bacterial Hybrid ATP-Binding Cassette Exporters: Molecular Recognition of the LipBCD, PrtDEF, and HasDEF Exporters

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Serratia marcescens **secretes several proteins, such as the lipase LipA, the metalloprotease PrtA, and the heme-binding protein HasA, which is required for heme acquisition, through two N-terminal signal peptide-independent systems that are classified as bacterial ATP-binding cassette (ABC) exporters. One is the ABC exporter for HasA, consisting of the ABC protein HasD, the membrane fusion protein (MFP) HasE, and the outer membrane protein (OMP) HasF. The second, composed of LipB (an ABC protein), LipC (an MFP), and LipD (an OMP), promotes secretion of LipA and PrtA in** ***Escherichia coli*** **recombinant clones. PrtA, which shows homology to the** ***Erwinia chrysanthemi*** **metalloproteases, is efficiently secreted by** ***E. coli*** **cells carrying the** ***E. chrysanthemi*** **ABC exporter PrtD (ABC protein)-PrtE (MFP)-PrtF (OMP). The existence of distinct systems in this bacterium and of various substrates for these systems allowed the study of protein secretion by heterologous Has, Lip, and Prt systems and by Has-Lip and Lip-Prt hybrid exporters in the genuine host as well as in** ***E. coli*****. For that purpose,** ***lipB*****-,** ***lipC*****-, and** ***lipD*****-deficient mutants were isolated from** ***S. marcescens*** **8000 and their secretion of LipA and PrtA was analyzed. This demonstrated that a unique exporter, the Lip apparatus, in** ***S. marcescens*** **secretes both LipA and PrtA. Hybrid exporters were tested for secretion of HasA and LipA. The LipB-HasE-HasF exporter allowed secretion of LipA but not HasA, showing that the ABC protein LipB is responsible for the substrate specificity. LipA, HasA, and** ***E. chrysanthemi*** **PrtC were secreted via heterologous exporters and via some hybrid exporters. Analysis of secretion via hybrid exporters showed that specific interactions occur between MFPs and OMPs in these systems. These genetic experiments demonstrated that specific interactions between the ABC protein and the MFP are required for the formation of active exporters.**

In gram-negative bacteria, many proteins are secreted through both the inner and outer membranes, directly from the cytoplasm into the surrounding medium, without any detectable periplasmic intermediate (31, 41). These proteins lack an N-terminal signal sequence, and their secretion mechanism is independent of the *sec* gene-mediated pathway (37).

This transport process requires three specific components (11). The first is situated in the inner membrane and belongs to the well-characterized ATP-binding cassette (ABC) protein superfamily of transporters, which includes eukaryotic and prokaryotic proteins involved in the import or export of a wide variety of substrates, such as antibiotics, sugars, amino acids, peptides, and proteins (21). The second could be associated with both the inner and outer membranes and is a member of a novel family of transport accessory proteins, found mostly in gram-negative bacteria, which function in conjunction with membrane transporters such as the ABC proteins or the drug resistance proton-linked antiporters. They are involved in the export of peptides, proteins, drugs, metallic cations, and oligosaccharides. This protein family was recently termed the membrane fusion protein (MFP) family (12). The third component is an outer membrane protein (OMP) with a typical N-terminal signal sequence. The export system consisting of these three proteins is categorized as the ABC transporter family.

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The ABC exporters of gram-negative bacteria are usually encoded by genes linked to the structural gene for the extracellular secretory polypeptide (17). For example, the *Escherichia coli hlyA* gene, coding for α-hemolysin, is adjacent to the *hlyB* and *hlyD* genes, which encode the ABC protein and MFP of the exporter, respectively (31). However, the TolC protein (42), which is the OMP of this exporter, is not encoded in this gene cluster. The four highly homologous metalloproteases of *Erwinia chrysanthemi* are also encoded by contiguous genes clustered with three genes coding for their common ABC exporter (Prt system), in the order PrtD (an ABC protein), PrtE (an MFP), and PrtF (an OMP) (25). In this case, the *prtF* gene, coding for an OMP, is included in this gene cluster.

Unusually, *Serratia marcescens* secretes several unrelated proteins, including the lipase LipA (2), the metalloprotease PrtA (35), and the heme-binding protein HasA, which is required for heme acquisition (28), via ABC exporters. In a previous report, we described the two ABC exporters of *S. marcescens* required for secretion of these proteins. One is the ABC exporter for the HasA protein, composed of HasD (an ABC protein) and HasE (an MFP). The genes encoding these proteins are linked to the *hasA* gene and constitute an iron-regulated operon (27, 29). Recently, we identified the gene from *S. marcescens* encoding the OMP of this system, *hasF*, and found that this gene is located separately from the *hasADE* gene cluster and is not iron regulated (7). Thus, the exporter for HasA secretion (the Has system) in *S. marcescens* consists of HasD-HasE-HasF.

Another ABC exporter of *S. marcescens* is encoded by the

lipBCD genes (3). When this exporter, composed of LipB (an ABC protein), LipC (an MFP), and LipD (an OMP), is reconstituted in *E. coli*, it promotes the secretion of LipA and PrtA. The *lipBCD* genes constitute an operon, but the *lipA* and *prtA* genes are not linked to this operon. Thus, two ABC exporters with different genetic organizations are present in *S. marcescens.*

The lipase belongs to a family of proteins secreted by ABC exporters in gram-negative bacteria, including *S. marcescens* (2, 3) and *Pseudomonas fluorescens* (15, 40). The LipB, LipC, and LipD proteins, components of the Lip system, show a high degree of homology (45 to 55%) to PrtD, PrtE, and PrtF, respectively, of *E. chrysanthemi*. The ABC protein and MFP of the Lip system, LipB and LipC, are 45 and 53% homologous to HasD and HasE of *S. marcescens*, respectively (3). The *S. marcescens* metalloprotease PrtA belongs to a family of exoproteases exported by ABC exporters and exhibits a high degree of sequence similarity to the four *E. chrysanthemi* metalloproteases (PrtA, PrtB, PrtC, and PrtG) (25) and to the *Pseudomonas aeruginosa* AprA protease (13, 14). The *S. marcescens* PrtA protein is efficiently secreted in *E. coli* recombinant strains by the *E. chrysanthemi* Prt system. Most of the exoproteins using ABC exporters have a C-terminal uncleaved secretion sequence (10, 30, 31). These signals are specific, being very similar in each exoprotein family, which allows efficient complementation within a family (16, 18, 19). Among proteases, lipases, and HasA, their sequences are less conserved but still display sufficient sequence similarity to allow heterologous complementation, with proteases being secreted by the Has exporter. On the other hand, the signals of toxins and proteases are dissimilar, and heterologous complementation between exporters belonging to these two families occurs with low efficiency.

In a previous report, substrate specificity was demonstrated to be defined by the ABC protein by using hybrid exporter systems with the components PrtD-PrtE-PrtF of *E. chrysanthemi* and HasD-HasE-TolC of *S. marcescens* reconstituted in *E. coli* (6). A simple system to identify the proteins bound to the substrate in vitro was based on substrate affinity chromatography (26). An ordered association between the protein substrates and the three exporter components was seen: the substrate recognizes the ABC protein, which interacts with the MFP, which in turn binds to the OMP of the ABC exporter. In vivo studies of hybrid exporters were also performed in these systems. HasE forms an active exporter with PrtF and TolC, whereas PrtE forms an active exporter with PrtF but not with TolC. In vitro studies of multiprotein complexes bound to affinity resins by means of the substrates confirmed these differences between HasE and PrtE (26). Such functional differences between two highly homologous proteins (HasE and PrtE) might be very useful to define domains on these proteins responsible for the specific interactions between the MFP and OMP. On the other hand, in vivo and in vitro studies have shown that HasD and PrtD can both interact with PrtE and HasE, forming active exporters and multiprotein complexes that bind to affinity resins by means of the substrates.

In the present work, the study of protein secretion by heterologous *S. marcescens* exporters and by Has-Lip and Lip-Prt hybrid exporters was undertaken in the original host species as well as in *E. coli* recombinant strains. For that purpose, *lipB*-, *lipC*-, and *lipD*-deficient mutants were isolated from *S. marcescens* 8000 and LipA secretion via hybrid ABC exporters was analyzed. This allowed confirmation that a single exporter in *S. marcescens*, the Lip apparatus, secreted both the LipA and PrtA proteins. The existence of nonfunctional hybrid Has-Lip and Lip-Prt exporters shows that a specific interaction between the MFP and OMP also takes place in these two systems. In vivo genetic experiments demonstrated that specific interactions between the ABC protein and MFP are required for the formation of active exporters.

TABLE 1. Plasmids used for construction of hybrid ABC exporters

Plasmid	Gene coding for:				Reference
	Secreted protein	ABC protein	MFP	OMP	
pRUW4		*prtD*	*prtE*	*prtF*	6
pPrtD/pACYC		*prtD*			6
pPrtE/pACYC			*prtE*		6
pPrtF/pACYC				*prtF*	6
pPrtDE/pACYC		*prtD*	*prtE*		6
pRUW48		*prtD*		*prtE*	6
pPrtEF/pACYC			*prtE*	*prtF*	6
pK150		*hasD*	*hasE*		6
pACYCHasD6		*hasD*			This study
pACYCHasE7			*hasE*		This study
pMWE121	*lipA*				3
pKHE65	*lipA*	*lipB*	*lipC*	*lipD*	3
pKHE652	*lipA*		*lipC*	*lipD*	3
pKHE653	*lipA*	*lipB*		*lipD*	3
pKHE654	*lipA*	*lipB*	*lipC*		3
pKHE657	*lipA*			*lipD*	This study
pKHE658	*lipA*		*lipC*		This study
pKHE659	*lipA*	*lipB*			This study
pSYC13	*hasA*	*hasD*			29
pSYC21	*hasA*		*hasE*		29
pSYC34	*hasA*	*hasD*	*hasE*		29
pSYC134	*hasA*				29

MATERIALS AND METHODS

Strains, plasmids, and media. *E. coli* K-12 DH5 (36) and JM109 (45) were used as hosts for the construction of plasmids. *S. marcescens* 8000, a wild-type strain of Sr41 (33), was used as a parent strain for mutagenesis. *S. marcescens* TT392 (38) deficient in a host restriction enzyme was employed for the modification of plasmid DNA with which *S. marcescens* cells were transformed. *E. coli* C600 *malPp*::*prtC* (6) was used for the detection of the secretion of the *E. chrysanthemi* metalloprotease PrtC. Luria-Bertani medium (LB) (9) and 2× YT extract (36) were used as rich *media*, and the latter was used for homologous recombination. For lipase production, the cells of *S. marcescens* were cultured at 30°C for 18 h in 500-ml flasks containing 60 ml of the lipase medium (1, 32), which consisted 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% $FeSO_4 \cdot 7H_2O$, 0.5% Tween 80 (polyoxyethylene sorbitan monooleate), and 0.1% Colorin 102 (Sanyo Chemical Industries, Kyoto, Japan) (pH 7.0). Modified tributyrin agar (LBG agar) plates (2) were used to isolate extracellular-lipase-deficient mutants of *S. marcescens*. Antibiotics were added at the following concentrations: ampicillin, 500 μg/ml; kanamycin, 200 μg/ml; or chloramphenicol, 170 μg/ml; and for *E. coli*, ampicillin, 200 μg/ml; kanamycin, 100 μg/ml; or chloramphenicol, 170 μg/ml. The plasmids used for construction of hybrid ABC exporters are listed in Table 1.

General methods. DNA manipulations were carried out according to standard procedures (36). Transformation of *E. coli* and *S. marcescens* was done as described previously (20, 38). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (24).

Enzyme assay. Lipase activity in culture medium was measured with a Lipase Kit S (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) (23). Units of lipase activity were expressed as micromoles of product formed per minute. Protease activity was measured by proteolysis of casein as described previously (34).

Construction of plasmids. Plasmids carrying the *lipBCD* genes were constructed as follows. The 4.5-kb *Bam*HI-*Hin*dIII DNA fragments encoding the *lipBC* and *lipC* genes from pKHE65 and pKHE652 (3) were inserted into the corresponding sites of pMW219 (43), producing pMWBC11 (*lipBC*) and pMWC12 (*lipC*), respectively. The 3.9-kb *Bam*HI-*Eco*RI fragment encoding the *lipB* gene from pMWBCD10 was introduced into the corresponding sites of pMW119 (43), resulting in pMWlipB15. The 2.6-kb *Sal*I-*Eco*RV fragment encoding the *lipD* gene from pKHE90 was ligated with *Sal*I- and *Sma*I-digested pHSG299 (39), creating pHSGVS4. Frameshift mutations were introduced into the *lipB*, *lipC*, and *lipD* genes by blunting the *Apa*I, *Sal*I, and *Hin*dIII restriction sites, respectively (3). The plasmids pKHE657 and pKHE658 were constructed

by introducing the mutation on the *lipC* and *lipD* genes, respectively, of pKHE652 (3). A frameshift mutation in the *lipD* gene of pKHE653 resulted in plasmid pKHE659 (3).

Plasmid pK150 (6), carrying the *hasDE* genes of *S. marcescens*, was digested with *Sph*I and self-ligated to remove the *Sph*I fragment encoding the C-terminal half of the HasE protein, with the resulting plasmid being called pACYCHasD6. The blunt-ended 4.5-kb *Nco*I fragment harboring the *hasE* gene was subcloned into the *Eco*RV site of pACYC184 (8) to produce pACYCHasE7.

Gene disruption by homologous recombination. The 2.0-kb *Eco*RI fragment of pMWBCD10 (3) containing the *lipD* gene was subcloned into the corresponding site of pUC19, generating pUClipD20. The 2.6-kb *Eco*RI-*Bam*HI *kan* DNA fragment of pKT240 carrying the kanamycin resistance gene (*kan*) (5) was filled in and inserted into the *Eco*52I site of pUClipD20, generating pUClipD-Km9 carrying the *lipD*::*kan* gene. Homologous recombination was carried out by a method described previously (22).

Isolation of the lipase secretion mutant. For the isolation of non-lipase-secreting mutants, *S. marcescens* cells were mutagenized with *N*-methyl-*N*′-nitroso-*N*-nitrosoguanidine as described previously (38). The mutagenized cells were grown on LBG agar plates, and the mutants lacking clear-halo formation were selected.

Western blot (immunoblot) analysis of lipase, metalloproteases, and HasA. Antisera produced against *S. marcescens* LipA and PrtA were described previously (1, 2). The cell lysates and the culture media were subjected to SDS-PAGE. The proteins were stained with Coomassie brilliant blue G-250 or electrophoretically transferred to an Immobilon P filter (Millipore). The blots were blocked by soaking them in 5% nonfat dry milk in phosphate-buffered saline and then incubated with anti-LipA or anti-PrtA antiserum (diluted 1:500 to 1:1,000). Signals were detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and the enhanced chemiluminescence system (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom).

For detection of PrtC and HasA, the immunoblots were incubated with anti-*E. chrysanthemi* PrtB and PrtC or anti-*S. marcescens* HasA antibodies and revealed as described previously (6).

RESULTS

Isolation of mutants deficient in extracellular-lipase production. To investigate the secretion mechanism of the Lip system LipB-LipC-LipD in *S. marcescens* cells, several mutants deficient in lipase secretion were isolated. The *lipD*-deficient mutant was obtained by homologous recombination. The kanamycin resistance gene was inserted in the *lipD* gene cloned on pUC19 (45) to disrupt LipD function, and the resultant plasmid, pUClipD-Km9, was introduced into *S. marcescens* cells. Two isolated mutants, strains MD55 and MD90, secreted neither LipA nor PrtA into the culture supernatant (data not shown). Secretion of both proteins was recovered by introduction of the *lipD* plasmid pKHE657. Thus, *lipD*-deficient mutants of *S. marcescens* were created.

Insertion of the marker gene into the *lipB* and *lipC* genes was polar on the downstream genes as described previously (28). *lipB*- and *lipC*-deficient mutants were isolated from mutagenized cells of strain 8000 (Table 2). The extracellular lipase and protease activities in culture media of 19 of the resulting mutants were tested. All the mutants were deficient in the extracellular lipase activity (less than 2% of that of strain 8000). The extracellular protease activities of 11 mutants were also markedly reduced, to less than 15% of that of strain 8000. Analysis of the extracellular proteins of the isolated mutants by SDS-PAGE and immunoblotting (Fig. 1) revealed three phenotypes: (i) $LipA^{sec+}$ $PrtA^{sec+}$ mutants (sec+ superscript indicates that the protein was secreted), producing both PrtA- and LipA-related proteins but lacking the lipase activity (mutants 214, 417, and 435); (ii) $LipA^{sec-}$ $PrtA^{sec+}$ mutants (sec− superscript indicates that the protein was not secreted), lacking the extracellular LipA but producing PrtA (mutants 212, 401, 413, 420, and 433); and (iii) $LipA^{sec-}$ $PrtA^{sec-}$ mutants, secreting neither LipA nor PrtA into the medium (mutants 203, 216, 405, 406, 409, 414, 415, 419, 422, 432, and 434) (Table 2). The residual extracellular protease activity of the $LipA^{sec-}$ $PrtA^{sec-}$ mutants may come from a serine protease produced by *S. marcescens* (44).

TABLE 2. Lipase and protease production by mutant strains

Strain	Extracellular enzyme activity[a]		Production of:			
			Extracellular protein[d]		Lipase	
	Lipase[b]	Protease[c]	Lipase	Protease	Intracellular[e]	Extracellular[f]
8000	31	190	+	+	+	+
203	0.027	41	−	−	+	−
212	<0.01	360	−	+	−	+
214	0.18	280	+	+	+	+
216	<0.01	12	−	−	+	−
401	0.017	135	−	+	−	+
405	<0.01	16	−	−	+	−
406	<0.01	8	−	−	+	−
409	<0.01	<4	−	−	+	−
413	<0.01	270	−	+	−	+
414	0.13	<4	−	−	+	−
415	<0.01	16	−	−	+	−
417	<0.01	100	+	+	−	+
419	<0.01	29	−	−	+	−
420	<0.01	180	−	+	−	+
422	<0.01	29	−	−	+	−
432	<0.01	16	−	−	+	−
433	<0.01	250	−	+	−	+
434	0.011	12	−	−	+	−
435	0.45	240	+	+	+	+

[a] The cells were cultured in lipase medium at 30°C for 18 h.
[b] Activity is in units per milliliter (10^3) of culture medium.
[c] Activity is in units per milliliter of cultured medium.
[d] Protein appearing in the supernatants of lipase medium, identified by immunoblot analysis with antisera produced against the *S. marcescens* lipase and metalloprotease. +, detected; −, not detected.
[e] Intracellular lipase activities of the cells cultured in lipase medium. +, >20 U/mg of protein; −, ≤20 U/mg of protein.
[f] Activity measured as clear-halo formation, on a tributyrin plate, of the recombinant cells carrying pMWE121. +, detected; −, not detected.

Analysis of complementation of the mutants by the *lipA* and *lipBCD* genes. Introduction of the *lipA* plasmid allowed the $LipA^{sec-}$ $PrtA^{sec+}$ and $LipA^{sec+}$ $PrtA^{sec+}$ mutants to form clear halos on tributyrin plates (Table 2), revealing the mutation causing LipA deficiency. LipA secretion was recovered by introducing the *lipBCD* genes into the $LipA^{sec-}$ $PrtA^{sec-}$ mutants (except for mutant 203), demonstrating mutations in the *lipBCD* genes (Table 2).

To identify mutations in the *lipBCD* genes of the $LipA^{sec-}$ $PrtA^{sec-}$ mutants, plasmids containing the *lipBC*, *lipB*, *lipC*, and *lipD* genes were introduced and the recombinant cells were tested for clear-halo formation (Table 3). Introduction of the *lipD* plasmid pHSGVS4 caused clear-halo formation in mutant 406. Two mutants, 414 and 422, produced clear halos after introduction of the *lipB* plasmid pMWlipB15. The *lipC* plasmid pMWC12 conferred a lipase-producing phenotype on seven mutants, 216, 405, 409, 415, 419, 432, and 434. The SDS-PAGE and immunoblotting analysis showed that clear-halo formation coincided with the secretion of LipA and PrtA (data not shown).

Thus, two *lipD*-deficient mutants were constructed by homologous recombination. Two *lipB*-deficient mutants (414 and 422), seven *lipC*-deficient mutants (216, 405, 409, 415, 419, 432, and 434), and one *lipD*-deficient mutant (406) were obtained by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment.

Secretion of the *S. marcescens* lipase LipA by hybrid ABC exporters of the LipB-LipC-LipD and the HasD-HasE-HasF systems. The LipA protein is efficiently secreted via the LipB-LipC-LipD ABC exporter of *S. marcescens*; to test whether the second ABC exporter, HasD-HasE-HasF, also promotes LipA

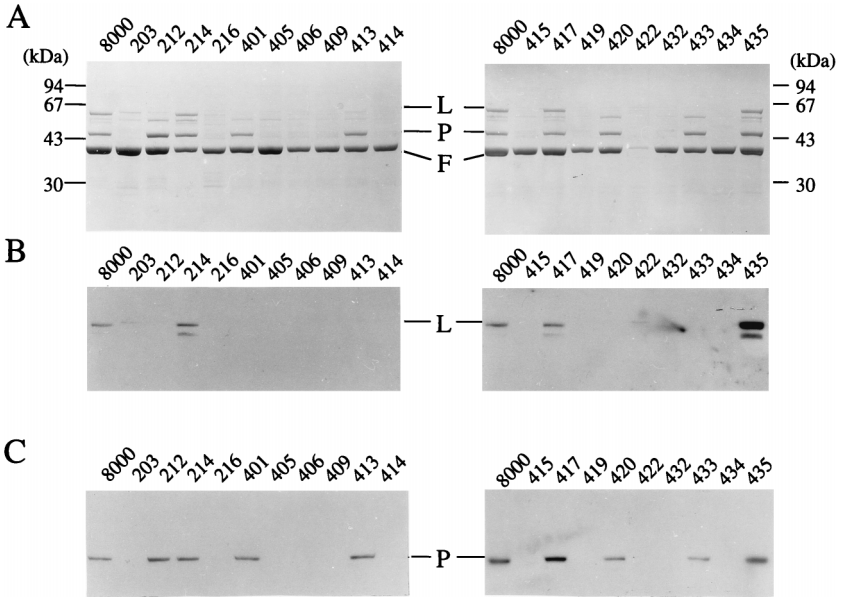

FIG. 1. Analysis of extracellular proteins of mutants deficient in extracellular lipase activity. Nineteen mutant strains were cultured in the lipase medium, and culture supernatants (0.15 optical density equivalent units) were subjected to SDS-PAGE (12.5% polyacrylamide). The gels were stained with Coomassie brilliant blue G-250 (A) and analyzed by immunoblotting with antiserum produced against *S. marcescens* LipA (B) or PrtA (C). Strain numbers are indicated above the gels. The positions of the LipA (L), PrtA (P), and flagellin proteins (F) (4) from *S. marcescens* are shown in the center. On either side of panel A are shown the positions of molecular mass markers.

secretion, culture supernatant of the *lipB* mutant 414 carrying pK150 encoding the *hasDE* genes was analyzed by immunoblotting (Fig. 2 and Table 4). It was revealed that secretion of LipA by the Has system is very inefficient (1%) in the original host, *S. marcescens*. The plasmids pACYCHasD6 and pACYCHasE7, carrying the *hasD* and *hasE* genes, respectively, were constructed from the plasmid pK150 (6). The plasmids pACYCHasD6, pACYCHasE7, and pK150 were introduced into the *lipB* mutant 414, the *lipC* mutant 415, and the *lipD* mutant 406, respectively, and the resultant recombinant cells were cultured in the lipase medium. The extracellular lipase activity was measured, and the proteins of culture supernatants were analyzed (Fig. 2 and Table 4). The LipA protein was detected in the cultured media of two recombinant strains, the *lipC* mutant harboring pACYCHasE7 and the *lipD* mutant carrying pK150 (Fig. 2). These recombinant strains produced the lipase at 33 and 28% of the level of the wild-type strain (Table 4). No lipase was secreted into the culture supernatant of the *lipB* mutant carrying pACYCHasD6 (Table 4 and Fig. 2). Thus, the LipB-HasE-HasF exporter promoted LipA secretion.

This hybrid exporter was also tested for HasA secretion by transforming the *lipC* mutant with pSYC21, a plasmid encoding HasA and HasE. The *lipC* mutant could not promote HasA secretion into iron-rich medium (Fig. 3). The HasD-LipC-LipD exporter was tested for HasA secretion in the *lipB* mutant transformed with a plasmid expressing HasA and HasD and grown in rich medium. HasA was not secreted. Hence, this

TABLE 3. Lipase secretion phenotypes of the Lip^{sec-} Prt^{sec-} mutants

Strain	Extracellular lipase activity with plasmid (gene)[a]:				
	pMWBCD10 (*lipBCD*)	pMWBC11 (*lipBC*)	pMWlipB15 (*lipB*)	pMWC12 (*lipC*)	pHSGVS4 (*lipD*)
203	−	−	−	−	−
216	+	+	−	+	−
405	+	+	−	+	−
406	+	−	−	−	+
409	+	+	−	+	−
414	+	+	+	−	−
415	+	+	−	+	−
419	+	+	−	+	−
422	+	+	+	−	−
432	+	+	−	+	−
434	+	+	−	+	−

[a] Activity measured as clear-halo formation, on a tributyrin plate, of the recombinant cells. +, detected; −, not detected.

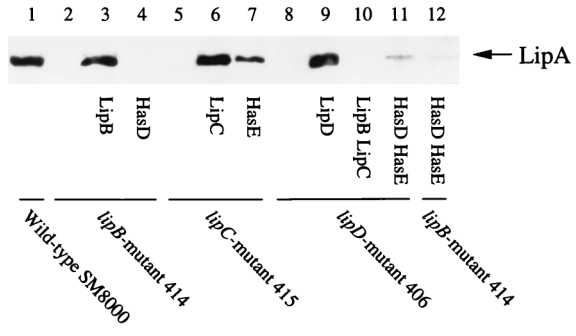

FIG. 2. Immunodetection of LipA in the supernatants of *S. marcescens* 8000, *lipB* mutant 414, *lipC* mutant 415, and *lipD* mutant 406 carrying *hasDE* plasmids. The recombinant strains were grown at 30°C in the lipase medium. The culture supernatants were analyzed by immunoblotting with antiserum produced against the *S. marcescens* lipase, as described in the legend to Fig. 1. The components introduced by plasmids are shown below each lane. Lanes: 1, *S. marcescens* (SM) 8000; 2, mutant 414 harboring pACYC184; 3, mutant 414 harboring pMWlipB15; 4, mutant 414 harboring pACYCHasD6; 5, mutant 415 harboring pACYC184; 6, mutant 415 harboring pMWC12; 7, mutant 415 harboring pACYCHasE7; 8, mutant 406 harboring pACYC184; 9, mutant 406 harboring pHSGVS4; 10, mutant 406 harboring pMWBC11; 11, mutant 406 harboring pK150; 12, mutant 414 harboring pK150.

TABLE 4. Extracellular lipase activities of the mutants carrying the various plasmids

Strain (relevant genotype)	Plasmid	Extracellular lipase activity (U/ml, 10^3)	ABC exporter components
8000 (wild type)	pACYC184	24	LipB LipC LipD HasF
414 (*lipB*)	pACYC184	<0.1	LipC LipD HasF
	pMWlipB15	22	LipB LipC LipD HasF
	pACYCHasD6	<0.1	LipC LipD HasD HasF
	pK150	0.22	LipC LipD HasD HasE HasF
	pPrtD/pACYC	<0.1	LipC LipD PrtD HasF
	pPrtDE/pACYC	<0.1	LipC LipD PrtD PrtE HasF
415 (*lipC*)	pACYC184	<0.1	LipB LipD HasF
	pMWC12	19	LipB LipC LipD HasF
	pACYCHasE7	7.9	LipB LipD HasE HasF
	pPrtE/pACYC	<0.1	LipB LipD PrtE HasF
	pPrtDE/pACYC	<0.1	LipB LipD PrtD PrtE HasF
	pPrtEF/pACYC	<0.1	LipB LipD PrtE PrtF HasF
406 (*lipD*)	pACYC184	<0.1	LipB LipC HasF
	pHSGVS4	28	LipB LipC LipD HasF
	pMWBC11	<0.1	LipB LipC HasF
	pK150	6.8	LipB LipC HasD HasE HasF
	pPrtF/pACYC	11	LipB LipC PrtF HasF
	pPrtDE/pACYC	<0.1	LipB LipD PrtD PrtE HasF

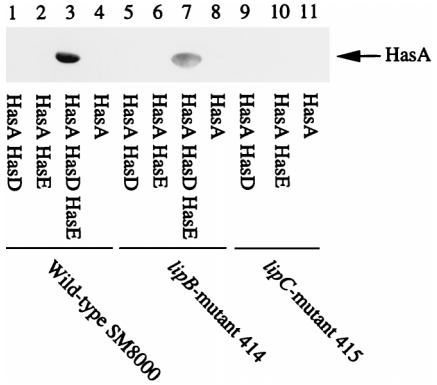

FIG. 3. Immunodetection of HasA in the supernatants of *S. marcescens* 8000, *lipB* mutant 414, and *lipC* mutant 415 carrying plasmids harboring different genes of the Has system. *S. marcescens* cells were grown at 30°C in iron-rich LB medium. Concentrated supernatants (0.5 optical density equivalents) were prepared when the bacteria reached early stationary phase, as described previously (6), and subjected to SDS-PAGE (15% polyacrylamide). The gel was analyzed by immunoblotting with antibody produced against *S. marcescens* HasA. The components introduced by plasmids are shown below the lanes. Lanes: 1, *S. marcescens* (SM) 8000 harboring pSYC13; 2, *S. marcescens* 8000 harboring pSYC21; 3, *S. marcescens* 8000 harboring pSYC34; 4, *S. marcescens* 8000 harboring pSYC134; 5, mutant 414 harboring pSYC13; 6, mutant 414 harboring pSYC21; 7, mutant 414 harboring pSYC34; 8, mutant 414 harboring pSYC134; 9, mutant 415 harboring pSYC13; 10, mutant 415 harboring pSYC21; 11, mutant 415 harboring pSYC134.

hybrid exporter, HasD-LipC-LipD, does not promote secretion of either LipA or HasA, suggesting that it is not functional.

Secretion of the *S. marcescens* lipase LipA and the *E. chrysanthemi* metalloprotease PrtC by hybrid ABC exporters of the LipB-LipC-LipD and the PrtD-PrtE-PrtF systems. The Lip system is homologous to the *E. chrysanthemi* Prt system, PrtD-PrtE-PrtF. We therefore tested secretion of LipA and PrtC by heterologous and hybrid exporters. The LipA and PrtC proteins are both secreted by heterologous exporters, with an efficiency ranging from 20 to 100% (Tables 4 and 5). LipB-LipC-PrtF was functional for LipA secretion in *E. coli* (Fig. 4) as well as *S. marcescens* (Fig. 5). It also promoted PrtC secretion, as shown in Fig. 6. This shows that LipC-like HasE can interact with one or the other OMP. On the other hand, PrtD-PrtE-LipD was not functional for either LipA secretion in *S. marcescens* (Fig. 5) or PrtC secretion in *E. coli* (Fig. 6). This shows that PrtE can interact only with PrtF, whereas HasE and LipC display a broader specificity and interact with OMPs of

TABLE 5. Secretion of *E. chrysanthemi* PrtC and *S. marcescens* LipA by bacterial hybrid ABC exporters reconstituted in *E. coli*

ABC exporter components			Estimated % secretion of[a]:	
ABC protein	MFP	OMP	PrtC	LipA
PrtD	PrtE	PrtF	100	20
PrtD	PrtE	LipD	<0.5	<0.5
LipB	LipC	LipD	20	100
LipB	LipC	PrtF	100	110
PrtD	LipC	PrtF	<0.5	<0.5
PrtD	LipC	LipD	<0.5	<0.5
LipB	PrtE	PrtF	2	<0.5
LipB	PrtE	LipD	<0.5	<0.5

[a] The estimated percent secretion is the ratio of the optical densities of the tested culture and the control (*E. coli* C600 harboring pKHE65 for LipA secretion or C600 *malPp*::*prtC* harboring pRUW4 for PrtC secretion) for the same secretion intensity.

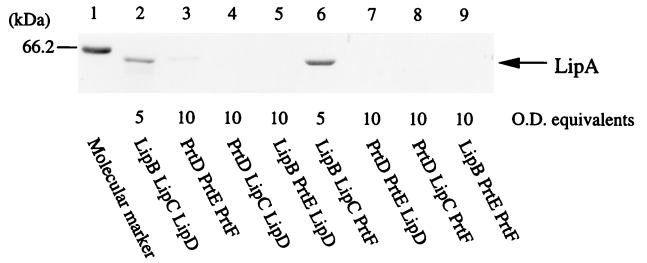

FIG. 4. Secretion of the *S. marcescens* lipase in *E. coli* by hybrid ABC exporters of the Lip and Prt systems. *E. coli* C6000 cells were grown at 37°C in LB medium overnight. Inactive concentrated supernatants were prepared by trichloroacetic acid precipitation as described previously (6). Samples of each supernatant containing various optical density (O.D.) equivalents were subjected to SDS-PAGE (10% polyacrylamide) and stained with Coomassie brilliant blue. The reconstitution pathway is shown below. Lanes: 1, molecular mass marker; 2, pKHE65; 3, pRUW4; 4, pPrtD/pACYC plus pKHE652; 5, pPrtE/pACYC plus pKHE653; 6, pPrtF/pACYC plus pKHE654; 7, pPrtDE/pACYC plus pKHE657; 8, pRUW48 plus pKHE658; and 9, pPrtEF/pACYC plus pKHE659.

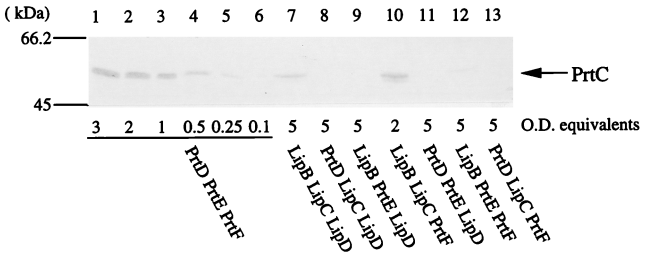

FIG. 6. Immunodetection of PrtC in culture supernatants of *E. coli* carrying hybrid ABC exporters of the Lip and Prt systems. Cultures of *E. coli* C600 *malPp*::*prtC* were grown at 37°C in LB medium. At an optical density of 0.5, PrtC synthesis was induced by adding maltose (to 0.1%) and isopropyl-β-D-thiogalactopyranoside (to 1 mM). Concentrated supernatants with various optical density (O.D.) equivalents were prepared when cells reached early stationary phase, as described previously (6), and subjected to SDS-PAGE (10% polyacrylamide). The gel was analyzed by immunoblotting with antibody produced against *E. chrysanthemi* PrtC. The positions of molecular mass markers are shown on the left. The components introduced by plasmids are shown below the lanes. Lanes: 1 to 6, pRUW4; 7, pKHE65; 8, pPrtD/pACYC plus pKHE652; 9, pPrtE/pACYC plus pKHE653; 10, pPrtF/pACYC plus pKHE654, 11, pPrtDE/pACYC plus pKHE657; 12, pPrtEF/pACYC plus pKHE659; 13, pRUW48 plus pKHE658.

several ABC exporters. Exchanges of inner membrane components were subsequently performed in exporters carrying the functional MFP-OMP pairs. We observed that PrtD cannot associate with LipC and that LipB-PrtE-PrtF forms a very inefficient exporter for both LipA and PrtA secretions.

DISCUSSION

S. marcescens possesses two types of protein exporters, the Has and Lip systems, which each consist of an ABC protein, an MFP, and an OMP and are classified as bacterial ABC exporters. The Lip system reconstituted in *E. coli* promotes secretion of LipA and PrtA but not HasA.

In the present work, *lipB*, *lipC*, and *lipD* mutants were isolated from *S. marcescens* and examined for secretion of LipA and PrtA (Fig. 1). This allowed us to demonstrate that a unique exporter in *S. marcescens*, the Lip apparatus, secretes both the LipA and PrtA proteins.

The Has system reconstituted in *E. coli* does not promote LipA secretion; reciprocally, the Lip system reconstituted in *E. coli* does not promote HasA secretion. Secretion of HasA and LipA by heterologous exporters was tested in a wild-type strain and in *lip* mutants of *S. marcescens* (Fig. 2 and 3). Plasmid-encoded HasA was not secreted by *S. marcescens* grown in rich medium (Fig. 3). LipA secretion by the *S. marcescens lipB* mutant carrying a plasmid containing the *hasDE* genes and grown in lipase medium was very inefficient (Fig. 2). This shows that there is no efficient complementation between these two systems. Has-Lip hybrid exporters were formed in recombinant cells of the *S. marcescens* mutants transformed with plasmids constitutively expressing the various *has* genes. LipA secretion was tested after induction of the chromosomal *lipA* gene in lipase medium. HasA secretion was tested in rich medium to avoid induction of the *has* operon in iron-depleted medium. The LipB-HasE-HasF exporter secretes LipA (Fig. 2) but not HasA (Fig. 3), showing that the ABC protein, LipB, is responsible for the substrate specificity in the natural host.

Similarly, the HasD-LipC-LipD exporter was tested for secretion of LipA and HasA in the *lipB* mutant transformed with a plasmid expressing HasA and HasD, or HasD alone, grown either in rich medium (HasA production) or in lipase medium (LipA production) (Fig. 2 and 3). Neither HasA nor LipA was secreted, suggesting that this hybrid is nonfunctional. Since LipC interacts with LipD, this result shows that HasD cannot combine with LipC to form an active exporter, suggesting the occurrence of a specific interaction between the ABC protein and MFP.

In the case of protein secretion via the Lip-Prt hybrid exporters (Fig. 4 to 6), LipB-LipC-PrtF was functional for secretion of LipA and PrtC but PrtD-PrtE-LipD was not functional for secretion of either of these proteins, showing that PrtE can interact only with PrtF whereas HasE and LipC display a broader specificity and interact with several OMPs (6). Exchanges of inner-membrane components were then performed in exporters carrying the functional MFP-OMP pairs. We observed that PrtD cannot associate with LipC and that LipB cannot associate with PrtE for both LipA and PrtC secretion.

This represents the first genetic evidence for in vivo "cross-talk" between the ABC protein and MFP, opening the possibility of studying hybrid proteins and defining the domains involved in such interactions.

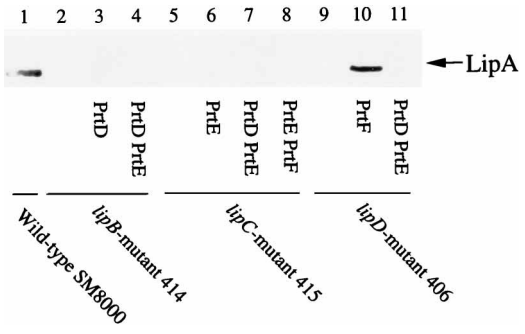

FIG. 5. Immunodetection of LipA in culture supernatants of *S. marcescens* 8000, *lipB* mutant 414, *lipC* mutant 415, and *lipD* mutant 406 carrying the *prtDEF* plasmids. The recombinant strains were grown at 30°C in the lipase medium, and culture supernatants were subjected to SDS-PAGE as described in the legend to Fig. 1. The gel was analyzed by immunoblotting with antiserum produced against the *S. marcescens* lipase. The components introduced by plasmids are shown below the lanes. Lanes: 1, *S. marcescens* 8000; 2, mutant 414 harboring pACYC184; 3, mutant 414 harboring pPrtD/pACYC; 4, mutant 414 harboring pPrtDE pACYC; 5, mutant 415 harboring pACYC184; 6, mutant 415 harboring pPrtE/pACYC; 7, mutant 415 harboring pPrtDE/pACYC; 8, mutant 415 harboring pPrtEF/pACYC; 9, mutant 406 harboring pACYC184; 10, mutant 406 harboring pPrtF/pACYC; 11, mutant 406 harboring pPrtDE/pACYC.

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REFERENCES

1. **Akatsuka, H., E. Kawai, K. Omori, S. Komatsubara, and T. Shibatani.** 1996. Overproduction of the extracellular lipase is closely related to that of metalloprotease in *Serratia marcescens*. J. Ferment. Bioeng. **81:**115–120.
2. **Akatsuka, H., E. Kawai, K. Omori, S. Komatsubara, T. Shibatani, and T. Tosa.** 1994. The *lipA* gene of *Serratia marcescens* which encodes an extracellular lipase having no N-terminal signal peptide. J. Bacteriol. **176:**1949–1956.
3. **Akatsuka, H., E. Kawai, K. Omori, and T. Shibatani.** 1995. The three genes *lipB*, *lipC*, and *lipD* involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. J. Bacteriol. **177:**6381–6389.
4. **Akatsuka, H., E. Kawai, K. Omori, and T. Shibatani.** 1995. Divergence of a flagellar protein in *Serratia marcescens*. Gene **163:**157–178.
5. **Bagdasarian, M. M., E. Amann, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1983. Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene **26:**273–282.
6. **Binet, R., and C. Wandersman.** 1995. Protein secretion by hybrid bacterial ABC-transporters: specific functions of the membrane ATPase and the membrane fusion protein. EMBO J. **14:**2298–2306.
7. **Binet, R., and C. Wandersman.** 1996. Cloning the *Serratia marcescens hasF* gene encoding the Has ABC exporter outer membrane component: a TolC analogue. Mol. Microbiol. **22:**265–273.
8. **Chang, A. C. Y., and S. N. Cohen.** 1977. Genome construction between bacterial species in vivo; replication and expression of *Staphylococcus* plasmid genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **71:**1030–1034.
9. **Davis, R. W., D. Bostein, and J. R. Roth.** 1980. Advanced bacterial genetics: a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Delepelaire, P., and C. Wandersman.** 1990. Protease secretion in gram-negative bacteria. J. Biol. Chem. **265:**17118–17125.
11. **Delepelaire, P., and C. Wandersman.** 1991. Characterization, localization and transmembrane organization of the three proteins PrtD, PrtE, and PrtF necessary for protease secretion by the gram-negative bacterium *Erwinia chrysanthemi*. Mol. Microbiol. **5:**2427–2434.
12. **Dinh, T., I. T. Paulsen, and M. H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.
13. **Duong, F.** 1994. La protease alcaline de *Pseudomonas aeruginosa*: modée d'étude de la sécréion des proteines par la voie ABC dépendante. Ph.D. thesis. Université d'Aix-Marseilles II, Marseilles, France.
14. **Duong, F., A. Lazdunski, B. Cami, and M. Murgier.** 1992. Nucleotide sequence of a cluster of genes involved in synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretion pathways. Gene **121:**47–54.
15. **Duong, F., C. Soscia, A. Lazdunski, and M. Murgier.** 1994. The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. Mol. Microbiol. **11:**1117–1126.
16. **Fath, M. J., R. C. Skvirsky, and R. Kolter.** 1991. Functional complementation between bacterial MDR-like export systems: colicin V, alpha-hemolysin, and *Erwinia* protease. J. Bacteriol. **173:**7549–7556.
17. **Gilson, L., H. K. Mahanty, and R. Kolter.** 1990. Genetic analysis of an MDR-like export system: the secretion of colicin V. EMBO J. **9:**3875–3884.
18. **Guzzo, J., F. Duong, C. Wandersman, M. Murgier, and A. Lazdunski.** 1991. The secretion genes of *Pseudomonas aeruginosa* alkaline protease are functionally related to those of *Erwinia chrysanthemi* proteases and *Escherichia coli* α-haemolysin. Mol. Microbiol. **5:**447–453.
19. **Guzzo, J., J.-M. Pages, F. Duong, A. Lazdunski, and M. Murgier.** 1991. *Pseudomonas aeruginosa* alkaline protease: evidence for secretion genes and study of secretion mechanism. J. Bacteriol. **173:**5290–5297.
20. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
21. **Higgins, C. F.** 1992. ABC transporters—from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
22. **Kiel, J. A. K. W., J. P. M. J. Vosson, and G. Venema.** 1987. A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation. Mol. Gen. Genet. **207:**294–301.
23. **Kurooka, S., S. Okamoto, and M. Hashimoto.** 1977. A novel and simple calorimetric assay for human serum lipase. J. Biochem. **81:**361–369.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **228:**680–685.
25. **Létoffé, S., P. Delepelaire, and C. Wandersman.** 1990. Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions are analogous to those of *Escherichia coli* α-haemolysin. EMBO J. **9:**1375–1382.
26. **Létoffé, S., P. Delepelaire, and C. Wandersman.** 1996. Protein secretion in gram-negative bacteria: assembly of the three components of ABC protein-mediated exporters is ordered and promoted by substrate binding. EMBO J. **15:**5804–5811.
27. **Létoffé, S., J.-M. Ghigo, and C. Wandersman.** 1993. Identification of two components of the *Serratia marcescens* metalloprotease transporter: protease SM secretion in *Escherichia coli* is TolC dependent. J. Bacteriol. **175:**7321–7328.
28. **Létoffé, S., J. M. Ghigo, and C. Wandersman.** 1994. Iron acquisition from heme and hemoglobin by a *Serratia marcescens* extracellular protein. Proc. Natl. Acad. Sci. USA **91:**9876–9880.
29. **Létoffé, S., J. M. Ghigo, and C. Wandersman.** 1994. Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. J. Bacteriol. **176:**5372–5377.
30. **Ludwig, A., T. Jarchau, R. Benz, and W. Goebel.** 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. Mol. Gen. Genet. **214:**553–561.
31. **Mackman, N., J.-M. Nicaud, L. Gray, and I. B. Holland.** 1986. Secretion of haemolysin by *Escherichia coli*. Curr. Top. Microbiol. Immunol. **125:**159–181.
32. **Matsumae, H., M. Furui, and T. Shibatani.** 1993. Lipase-catalyzed asymmetric hydrolysis of 3-phenylglycidic acid ester, the key intermediate in the synthesis of diltiazem hydrochloride. J. Ferment. Bioeng. **75:**93–98.
33. **Matsumoto, H., S. Hosogaya, K. Suzuki, and T. Tazaki.** 1975. Arginine gene cluster of *Serratia marcescens*. Jpn. J. Microbiol. **19:**35–44.
34. **Miyata, K., K. Maejima, K. Tomoda, and M. Isono.** 1970. *Serratia* protease. I. Purification and general properties of the enzyme. Agric. Biol. Chem. **34:**310–318.
35. **Nakahama, K., K. Yoshimura, R. Marumoto, M. Kikuchi, I. S. Lee, T. Hase, and H. Matsubara.** 1986. Cloning and sequencing of *Serratia* protease gene. Nucleic Acids Res. **14:**5843–5855.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Schatz, P. J., and J. Beckwith.** 1990. Genetic analysis of protein export in *Escherichia coli*. Annu. Rev. Genet. **24:**215–248.
38. **Takagi, T., and M. Kisumi.** 1985. Isolation of a versatile *Serratia marcescens* mutant as a host and molecular cloning of the aspartase gene. J. Bacteriol. **161:**1–6.
39. **Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh.** 1987. High-copy-number plasmid vectors for *lacZ* α-complementation and chloramphenicol- or kanamycin-resistant selection. Gene **61:**63–74.
40. **Tan, Y., and K. J. Miller.** 1992. Cloning, expression, and nucleotide sequence of a lipase gene from *Pseudomonas fluorescens* B52. Appl. Environ. Microbiol. **58:**1402–1407.
41. **Wandersman, C.** 1992. Secretion across the bacterial outer membrane. Trends Genet. **8:**317–321.
42. **Wandersman, C., and P. Delepelaire.** 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA **87:**4776–4780.
43. **Yamaguchi, K., and Y. Masamune.** 1985. Autogenous regulation of synthesis of the replication protein in plasmid pSC101. Mol. Gen. Genet. **200:**362–367.
44. **Yanagida, N., T. Uozumi, and T. Beppu.** 1986. Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli*. J. Bacteriol. **166:**937–944.
45. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.