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Exchange of Xcp (Gsp) Secretion Machineries between *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*: Species Specificity Unrelated to Substrate Recognition

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***Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* are gram-negative bacteria that secrete proteins using the type II or general secretory pathway, which requires at least 12 *xcp* gene products (XcpA and XcpP to -Z). Despite strong conservation of this secretion pathway, gram-negative bacteria usually cannot secrete exoproteins from other species. Based on results obtained with *Erwinia*, it has been proposed that the XcpP and/or XcpQ homologs determine this secretion specificity (M. Linderberg, G. P. Salmond, and A. Collmer, Mol. Microbiol. 20:175–190, 1996). In the present study, we report that XcpP and XcpQ of *P. alcaligenes* could not substitute for their respective *P. aeruginosa* counterparts. However, these complementation failures could not be correlated to species-specific recognition of exoproteins, since these bacteria could secrete exoproteins of each other. Moreover, when *P. alcaligenes xcpP* and *xcpQ* were expressed simultaneously in a *P. aeruginosa xcpPQ* deletion mutant, complementation was observed, albeit only on agar plates and not in liquid cultures. After growth in liquid culture the heat-stable *P. alcaligenes* XcpQ multimers were not detected, whereas monomers were clearly visible. Together, our results indicate that the assembly of a functional Xcp machinery requires species-specific interactions between XcpP and XcpQ and between XcpP or XcpQ and another, as yet uncharacterized component(s).**

Many extracellular proteins produced by gram-negative bacteria are secreted by the type II secretion pathway, also called the main terminal branch (MTB) of the general secretory pathway (GSP) (49). *Pseudomonas aeruginosa* secretes several enzymes and toxins via this pathway (20). These proteins are translocated in two steps across the bacterial cell envelope. After translocation of the signal peptide-bearing exoproteins across the cytoplasmic membrane, a step similar to the Sec-mediated transport of proteins in *Escherichia coli* (16, 51), the exoproteins are transported from the periplasm across the outer membrane. The latter process requires the products of at least 12 *xcp* genes (20). The *xcpA* gene is located between positions 5072694 and 5073566 of the chromosomal map, and *xcpPQRSTUVWXYZ* are located between positions 3475955 and 3483641 (http://www.pseudomonas.com). Homologs of *xcp* genes, encoding components of the MTB of the GSP, are present in many other gram-negative bacteria (10, 18, 20) and are now usually referred to as *gsp* genes. In addition, homologs of several *xcp* gene products are involved in other macromolecular transport processes, such as the formation of type IV pili, filamentous phage assembly, type III protein secretion, and natural uptake of DNA (23, 32).

Xcp (Gsp) proteins are located in the cell envelope and are proposed to interact and to function as a protein secretion apparatus called the secreton. The only outer membrane component of the secreton is XcpQ (GspD) (2). All the other Xcp proteins are located in or associated with the cytoplasmic membrane. XcpQ (2) and homologs (8, 28, 37, 39, 55) belong to a new family of proteins called secretins, which form multimers in the outer membrane. These multimers may form a channel with a large central cavity through which the exoproteins are likely to pass (7, 43, 46). Moreover, XcpQ has been proposed to interact with bitopic inner membrane protein XcpP (GspC) (3).

It remains unclear how exoproteins, which are at least partially folded before secretion (5, 6, 21, 47, 50), are distinguished from periplasmic proteins. A putative secretion motif involved in this process might be present within the three-dimensional structure of the exoproteins (42, 53, 57). Moreover, recognition of the exoproteins by the Gsp secreton appears to be species specific. Although secretion of an exoprotein expressed in a heterologous host has occasionally been reported (45, 58), it does not generally occur (10, 45), not even with similar enzymes from the closely related bacterial species *Erwinia carotovora* and *Erwinia chrysanthemi* (30, 52). The last of these studies also suggested that the XcpP (GspC) and XcpQ (GspD) homologues are involved in this species specificity, since, except for *gspC* and *gspD*, every *gsp* gene of *E. chrysanthemi* could be replaced by its counterpart from *E. carotovora*, (41). Moreover, it was demonstrated that GspD from *E. chrysanthemi* interacts with exoproteins from *E. chrysanthemi* but not with those from *E. carotovora* (55).

Recently, the *xcp* gene cluster of *Pseudomonas alcaligenes* was cloned and characterized (25). As in *P. aeruginosa*, the *xcp*

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TABLE 1. Bacterial strains

Strain	Relevant characteristics	Source or reference
P. aeruginosa		
PAO1	Prototroph	Holloway collection
DZQ40	PAO1 Δ*xcpP-Z*	1
PAO1ΔPQ	PAO1 Δ*xcpPQ*	This study
PAO1ΔRZ	PAO1 Δ*xcpR-Z*	V. Chapon-Hervé
PAO1ΔP	PAO1 Δ*xcpP*	3
PAG2	PAO1 Δ*xcpQ*	This study
PAG3	PAO1 Δ*xcpRS*	This study
PAO1ΔT	PAO1 Δ*xcpT*	V. Chapon-Hervé
PAO1ΔX	PAO1 Δ*xcpX*	4
KS910-503	PAO503 *xcpY51*	59
KS902-503	PAO503 *xcpZ5*	59
PABS1	PAO1 Δ*lipAH*	K.-E. Jaeger
P. alcaligenes		
Ps93	Restriction-negative modification-positive strain M-1 mutant	24
Ps93R	Ps93 *xcpR*::Km^r	This study
E. coli		
CC118(λ*pir*)	Strain to maintain pKNG101	31
PC2494	*hsdR thi* Δ(*lac-proAB*) *supE* (F′ *proA*$^+$*B*$^+$ *lacI*^{q}*Z*ΔM15 *traD36*)	Phabagen collection[a]

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genes of *P. alcaligenes* are organized in two divergently transcribed operons, *xcpPQ* and *xcpR* to *-Z*. The degree of homology between the *xcp* gene products of *P. alcaligenes* and *P. aeruginosa* ranges from 42 to 82% amino acid identity, which is comparable to the degree of homology between the Gsp proteins of *E. chrysanthemi* and *E. carotovora* (41).

The goal of the present study was to determine whether species specificity of secretion between *P. aeruginosa* and *P. alcaligenes* exists. Therefore, the ability of these bacteria to secrete exoproteins of each other was tested. We also investigated whether individual Xcp proteins from *P. alcaligenes* and *P. aeruginosa* can functionally be exchanged. We demonstrate the species specificity of the XcpP and XcpQ proteins, but, in contrast to what was found for *Erwinia*, this specificity is not related to substrate recognition.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Tables 1 and 2, respectively. Strains were grown at 37°C in Luria broth (LB) or tryptic soy broth (TSB) with agitation or on LB agar plates. Plasmids were introduced into *P. aeruginosa* and *P. alcaligenes* by electroporation or by conjugation using pRK2013 as a helper plasmid in triparental matings. Antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 100 (*E. coli*); carbenicillin, 100 (*P. alcaligenes*) or 300 (*P. aeruginosa*); tetracycline, 5 (*P. alcaligenes*), 15 (*E. coli*), or 100 (*P. aeruginosa*); kanamycin, 10 (*P. alcaligenes*), 25 (*E. coli*), or 400 (*P. aeruginosa*); streptomycin, 100 (*E. coli*) or 1,000 (*P. aeruginosa*). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at concentrations up to 2 mM when required.

Construction of *xcp* mutant strains. Plasmid pUAWB5, which contains a 5-kb *Bam*HI fragment, was used to construct a deletion of a 1.9-kb *Eco*RI fragment in *xcpQ*. Starting from the 6-kb *Eco*RI fragment in pUAWE6, a plasmid with a 1.4-kb *Sca*I-*Sma*I deletion in *xcpR* combined with a 1.1-kb *Asu*II deletion in *xcpS* was constructed from pEMBL19. The plasmids carrying the deletions were cloned in suicide vector pKNG101. These pKNG101 derivatives were introduced in PAO1, and deletion mutants resulting from double-crossover events were obtained as described previously (12, 36). A 1-kb *Eco*RI/*Hin*dIII DNA fragment containing an internal 500-bp deletion in the *xcpP* gene and a 881-bp *Xho*I/*Eco*RI DNA fragment containing the 3′ end of the *xcpQ* gene were cloned in tandem in pUC19, yielding pSB96. Plasmid pSB96 was introduced into PAO1, and a double-crossover event, yielding the *xcpPQ* deletion, was selected after screening for secretion-defective clones on skim milk plates. Secretion in mutants PAG2, PAG3, and PAO1ΔPQ could be restored by introducing plasmids carrying only *xcpQ*, *xcpRS*, and *xcpPQ*, respectively.

To obtain a *P. alcaligenes*, *xcpR* mutant, the kanamycin resistance gene of pUC4K was cloned as a *Hin*cII fragment into *xcpR* on plasmid pXA2, which was digested with both *Sma*I and *Eco*RV to delete a 0.9-kb fragment of *xcpR*. The resulting plasmid was introduced into strain Ps93, and colonies resistant to kanamycin but sensitive to carbenicillin, were selected. Defective secretion of lipase was verified on plates containing tributyrin.

SDS-PAGE and immunoblotting. Cells were harvested at optical densities at 600 nm (OD_{600}) of 3 to 4. Proteins were precipitated from the supernatants with 5% (wt/vol) trichloroacetic acid (final concentration). Cellular and extracellular proteins were solubilized in sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels contained 11% acrylamide, unless otherwise stated. Immunoblots were incubated with appropriate polyclonal or monoclonal antibodies and subsequently with peroxidase-conjugated secondary antibodies, followed by detection by chemiluminescence (Pierce).

Enzyme assays. Plates for the detection of protease activity contained 1.5% skim milk. Elastase activity was analyzed on plates with a top layer containing 1% elastin. Quantitative assays for elastase were performed as described previously (10). Plates for the detection of lipase activity contained 1% tributyrin, which was added from a 10× stock solution (10% tributyrin emulsified with 8% gum arabic by short sonication). Quantitative assays for lipase were performed as described by Kordel and collaborators (38). One unit of lipase activity was defined as the amount of enzyme that liberated 1 nmol of *p*-nitrophenol from *p*-nitrophenyl palmitate per min. Lipase units were standardized to the activity contained in the supernatant of 1 OD_{600} unit equivalent of bacterial cell culture.

RESULTS

Heterologous secretion in *Pseudomonas* species. Related *Pseudomonas* species *P. aeruginosa* and *P. alcaligenes* were chosen to study heterologous protein secretion. Elastase (LasB) is a *P. aeruginosa* type II-secreted exoprotein which possesses a strong proteolytic activity. Plasmid pPB29, carrying the elastase structural gene (*lasB*), was introduced into *P. alcaligenes* strain Ps93. Immunoblotting revealed that the majority of the elastase produced by *P. alcaligenes* Ps93(pPB29) was present in the extracellular medium (Fig. 1). This secretion was Xcp dependent, since the majority of elastase produced by an *xcpR* mutant derivative of Ps93, strain Ps93R, was present in

TABLE 2. Plasmids

Plasmid	Relevant characteristics[a]	Source or reference
pEMBL18, -19	Ap^r; phagemid	14
pUC19	Ap^r; ColE1 *lacI* ϕ80d*lacZ*	60
pMMB67EH, -HE	IncQ Ap^r; *tac* promoter; *lacI*^{q}	22
pLAFR3	IncP Tc^r	56
pVDZ′2	IncP Tc^r; *lac* promoter	15
pKNG101	Sm^r; *oriR6K sacBR mobRK2*	36
pRK2013	Km^r; *ori* ColE1; Tra$^+$ Mob$^+$	17
pUC4K	Km^r gene block in pUC4	Pharmacia
pPB29	*lasB* on an *Eco*RI-*Hin*dIII fragment in pVDZ′2	This study
pJRDlipAB	*P. alcaligenes lipAB* in pJRD215	24
pAX24	*P. aeruginosa xcpP* to *-Z* cluster in pLAFR3	19
pUAWB5	*P. aeruginosa xcpPQ* on a 5.2-kb *Bam*HI fragment in pUC19	This study
pUAWE6	*P. aeruginosa xcpPRSTUV* in pUC19	10
pSB10	*P. aeruginosa xcpP* in pMMB67HE	3
pSB96	1.88-kb DNA fragment from the *P. aeruginosa xcp* cluster with an internal *xcpPQ* deletion in pUC19	This study
pMB4	*P. aeruginosa xcpQ* on a 2.9-kb *Pst*I-*Sma*I fragment in pMMB67HE	This study
pAF2	*P. aeruginosa xcpPQ* on a 5.2-kb *Bam*HI fragment in pLAFR3	This study
pLAF600	*P. alcaligenes xcpP* to *-Z* cluster in pLAFR3	25
pLAF600SB	*P. alcaligenes xcpPQ* in pLAFR3	25
pLAF600BH	*P. alcaligenes xcpR* to *-Z* in pLAFR3	25
pXA2	*P. alcaligenes xcpR* to *-V* on a 5.3-kb *Nco*I fragment in pEMBL18	This study
pMPA2	*P. alcaligenes xcpP* on a 1.0-kb *Sal*I-*Eco*RI fragment in pMMB67HE	This study
pCK28	*P. alcaligenes xcpQ* on a 2.6-kb *Bsa*AI-*Sal*I fragment in pMMB67EH	This study
pRVA4	*P. alcaligenes xcpR* to *-V* on a 5.3-kb *Nco*I fragment in pMMB67HE	This study
pMTA3	*P. alcaligenes xcpT* on a 1.2-kb *Sph*I-*Xho*I fragment in pMMB67HE	This study
pMXA8	*P. alcaligenes xcpX* on a 1.6-kb *Sph*I-*Stu*I fragment in pMMB67HE	This study
pMYA3	*P. alcaligenes xcpY* on a 1.6-kb *Xho*I-*Sph*I fragment in pMMB67EH	This study
pMZA3	*P. alcaligenes xcpZ* on a 1.5-kb *Sma*I-*Eco*RI fragment in pMMB67HE	This study

[a] Tc^r, tetracycline resistant; Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Sm^r, streptomycin resistant.

the cellular fraction (Fig. 1). Similar conclusions were reached after plating the strains on skim milk plates. On such plates, no endogenous extracellular protease activity was detected with *P. alcaligenes* Ps93. However, large halos of degraded milk proteins were visible when strain Ps93(pPB29) was tested, but not when *xcpR* mutant Ps93R(pPB29) was tested (results not shown). It can thus be concluded that elastase from *P. aeruginosa* is secreted by the heterologous host *P. alcaligenes* in an Xcp-dependent manner.

Next, we assessed the ability of *P. aeruginosa* to secrete a *P. alcaligenes* Xcp-dependent exoprotein, i.e., lipase. Plasmid pJRDlipAB, containing the *P. alcaligenes* lipase gene (*lipA*) and the gene encoding its dedicated chaperone, LipB, was introduced into *P. aeruginosa* strains PAO1 (wild type), isogenic *lipAH* mutant PABS1, which is unable to produce the endogenous lipase and its chaperone, and *xcpP* to *-Z* deletion mutant DZQ40. The resulting strains were tested for lipase production on plates containing tributyrin as the substrate. For PAO1 and PABS1, but not DZQ40, clear halos revealing extracellular lipase activity were detected (Fig. 2A). When pJRDlipAB was not present in these strains, such halos were not formed (data not shown). After growth of the pJRDlipAB-containing strains in TSB medium and immunoblotting with monoclonal antibodies directed against *P. alcaligenes* lipase, this lipase was found to be secreted by strains PAO1 and PABS1 but not by DZQ40 (Fig. 2C). The specific lipase activities detected in the supernatant of these strains were 252, 249, and 0 U, respectively. When PAO1 lacking pJRDlipAB was grown in TSB medium, no *P. alcaligenes* lipase-specific protein could be detected in the supernatant (Fig. 2C) and only weak activity (12 U), due to the endogenous *P. aeruginosa* lipase, was observed. Hence, we concluded that (i) the high level of lipase activity observed is due to *P. alcaligenes* lipase and not to the endogenous *P. aeruginosa* lipase and (ii) *P. alcaligenes* lipase secretion in *P. aeruginosa* is Xcp dependent.

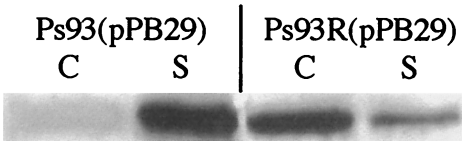

FIG. 1. Immunoblot demonstrating Xcp-dependent heterologous secretion of elastase by *P. alcaligenes*. Plasmid pPB29 containing the *lasB* gene encoding elastase of *P. aeruginosa* was introduced into *P. alcaligenes* Ps93 (Xcp$^+$) and into its *xcpR* mutant derivative Ps93R. Cells (C) and supernatant (S) were separated and analyzed by immunoblotting with an antiserum directed against elastase. Cellular and extracellular proteins corresponding to equal culture volumes were loaded.

Functional exchange of the Xcp machinery is dependent on growth conditions. The results of the heterologous secretion experiments demonstrate that the Xcp machineries of *P. aeruginosa* and *P. alcaligenes* are not species specific with respect to the recognition of each other's exoproteins. This lack of species specificity was further analyzed in complementation experiments in which *xcp* genes from both species were exchanged. To study whether the XcpP to -Z proteins from *P. alcaligenes* (XcpP-Z_{alc}) could assemble into a functional secreton in *P. aeruginosa*, cosmid pLAF600 carrying the *P. alcali-*

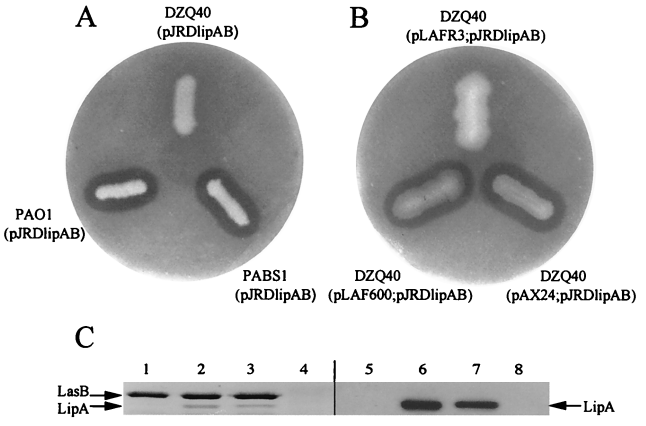

FIG. 2. Secretion of *P. alcaligenes* lipase by *P. aeruginosa*. (A and B) Plate assays demonstrating lipase secretion. (A) Plasmid pJRDlipAB, carrying the genes encoding *P. alcaligenes* lipase and its lipase-specific foldase, was introduced into *P. aeruginosa* strains PAO1, *xcpP* to *-Z* deletion mutant DZQ40, and *lipAH* deletion mutant PABS1. (B) Plasmid pJRDlipAB was cointroduced with pLAFR3, pAX24 carrying the *P. aeruginosa xcpP* to *-Z* cluster, or pLAF600 carrying the *P. alcaligenes xcpP* to *-Z* cluster into strain DZQ40. (C) Coomassie brilliant blue-stained gel (lanes 1 to 4) and immunoblot with a monoclonal antibody directed against *P. alcaligenes* lipase (lanes 5 to 8), using supernatants from *P. aeruginosa* strains grown in TSB medium. Lanes 1 and 5, PAO1; lanes 2 and 6, PAO1/pJRDlipAB, lanes 3 and 7, PABS1/pJRDlipAB; lanes 4 and 8, D40ZQ/pJRDlipAB. The positions of the *P. aeruginosa* elastase (LasB) and the *P. alcaligenes* lipase (LipA) are indicated.

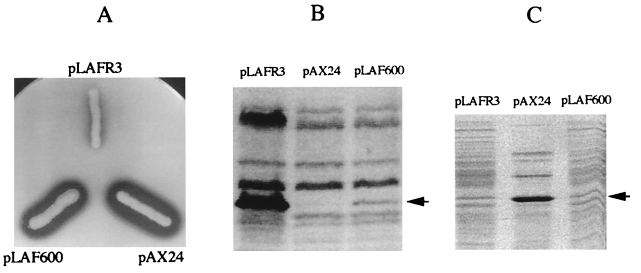

FIG. 3. Complementation of the *xcpP* to *-Z* deletion in *P. aeruginosa* strain DZQ40 by the *P. alcaligenes xcpP* to *-Z* cluster. Strain DZQ40 contained either pLAFR3, pAX24 carrying the *P. aeruginosa xcp* gene cluster, or pLAF600 carrying the *P. alcaligenes xcp* gene cluster. (A) Plate assay demonstrating protease secretion. (B) Immunoblot showing accumulation of elastase inside the cells. Strains were grown on a plate, resuspended in LB medium in order to measure the cell density, pelleted, and resuspended in sample buffer. Equal amounts of cells were loaded and analyzed with an antiserum directed against elastase (arrow). (C) Coomassie brilliant blue-stained gel showing proteins of culture supernatants. The major protein in the pAX24-labeled lane (arrow) is elastase.

genes xcpP to *-Z* genes was introduced into the *P. aeruginosa xcpP* to *-Z* deletion mutant DZQ40. DZQ40 strains containing cloning vector pLAFR3 and cosmid pAX24, carrying the *P. aeruginosa xcpP* to *-Z* gene cluster, were included as controls. Protease plate assays indicated that secretion of elastase was restored efficiently by the *P. alcaligenes xcp* gene cluster, since the halo size was almost identical to the one of strain DZQ40(pAX24) (Fig. 3A). Comparable results were obtained on plates containing elastin as the elastase substrate (results not shown). The halos observed could have been the result of secretion of only a small fraction of the total amount of elastase produced. To test this possibility, cells were collected after growth on plates and analyzed by immunoblotting with an antiserum directed against elastase. The results showed that only small amounts of elastase accumulated inside the cells of strain DZQ40 containing pLAF600, compared with the amounts inside cells of DZQ40 containing vector pLAFR3 (Fig. 3B), strongly suggesting that the majority of the elastase is secreted by strain DZQ40(pLAF600). The *P. alcaligenes xcpA* gene, encoding the prepilin peptidase, is not present on pLAF600 but is probably located separate from the *xcpP* to *-Z* genes as it is in *P. aeruginosa*. In line with this, a *P. aeruginosa xcpA* mutant containing pLAF600 did not show halo formation on skim milk plates (results not shown). These data indicate that the halo observed with strain DZQ40(pLAF600) is due to Xcp_{alc} -dependent secretion of elastase and not to non specific leakage. Finally, strain DZQ40(pLAF600) was able to grow on the selective lipid agar plates described by Kagami et al. (35) (results not shown), indicating that lipase, another Xcp-dependent *P. aeruginosa* exoprotein, was also secreted. Therefore, the *P. alcaligenes* secreton is functionally assembled in *P. aeruginosa*.

Complementation was also studies after growth of the same set of bacterial strains in liquid medium. Surprisingly, the results differed substantially from those of the plate assays. Compared to that of strain DZQ40(pAX24), the supernatant of strain DZQ40(pLAF600) contained hardly any elastase (Fig. 3C). Similarly, pJRDlipAB-encoded *P. alcaligenes* lipase was apparently secreted efficiently by *P. aeruginosa* strain DZQ40 expressing the *P. alcaligenes xcp* genes from pLAF600 if secretion was assessed on tributyrin plates (Fig. 2B), but not when culture supernatants were analyzed on blots (results not shown). However, when pJRDlipAB was introduced in DZQ40(pAX24), lipase secretion via the *P. aeruginosa* secreton was revealed under both growth conditions (Fig. 2B and data not shown). In conclusion, these results indicate that efficient functioning of the *P. alcaligenes* secreton is dependent on (i) the bacterial context in which the secreton is expressed and (ii) the growth conditions, i.e., on a plate or in liquid medium. These different situations are depicted in Fig. 4.

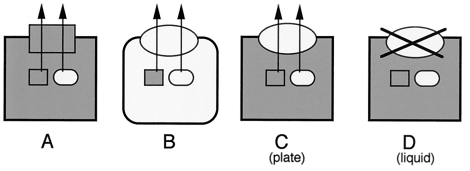

FIG. 4. Summary showing the dependency of heterologous secretion on the bacterial context in which the *xcp* genes are expressed and on the growth conditions. Rectangular and oval symbols represent cells, Xcp machineries, or exoproteins from *P. aeruginosa* and *P. alcaligenes*, respectively. When *xcp* genes are expressed in the natural host (A and B), both endogenous and heterologous exoproteins are secreted. However, when the *P. alcaligenes xcp* genes are expressed in *P. aeruginosa*, efficient secretion of either *P. alcaligenes* lipase or *P. aeruginosa* elastase occurs only when the strain is grown on a plate (C), not in liquid medium (D), possibly because of a secreton assembly problem in the latter conditions.

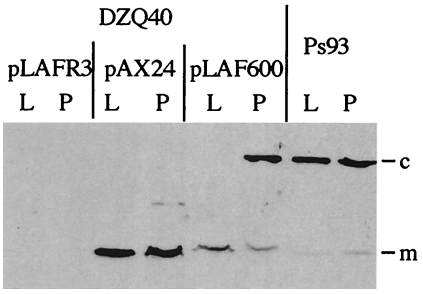

FIG. 5. Formation of a heat-stable multimeric complex by *P. alcaligenes* XcpQ protein. Cellular proteins, heated for 10 min at 95°C prior to SDS-PAGE, were analyzed by immunoblotting with an antiserum directed against *P. aeruginosa* XcpQ. *P. alcaligenes* strain Ps93 and derivatives of *P. aeruginosa xcpP* to *-Z* deletion mutant DZQ40 containing either pLAFR3, pAX24 carrying the *P. aeruginosa xcpP* to *-Z* gene cluster, or pLAF600 carrying the *P. alcaligenes xcpP* to *-Z* gene cluster were grown on a plate (P) or in liquid medium (L). Proteins were separated on a gel containing 3 and 9% acrylamide in the stacking and running gels, respectively. c, multimeric complex of *P. alcaligenes* XcpQ; m, XcpQ monomer.

Formation of *P. alcaligenes* XcpQ multimers in *P. aeruginosa*. The possibility that the *xcp* genes of *P. alcaligenes* were not expressed in *P. aeruginosa* DZQ40 during growth in liquid cultures was investigated by immunoblotting using antisera directed against *P. aeruginosa* Xcp proteins. The XcpR and XcpY proteins were detected in cell extracts of strain DZQ40(pLAF600), but not in those of strain DZQ40(pLAFR3), after growth both on a plate and in liquid medium (data not shown), demonstrating that *xcpR* and *xcpY* from *P. alcaligenes*, and very likely also the other genes in the *xcpR* to *-Z* operon, were expressed under both growth conditions.

P. aeruginosa XcpQ protein ($XcpQ_{aer}$) forms multimeric ring-shaped structures that dissociate upon heating in SDS-sample buffer (2). In contrast, the XcpQ multimers from *P. alcaligenes* are heat stable (7). To test the expression and oligomerization of $XcpQ_{alc}$ in *P. aeruginosa*, derivatives of strain DZQ40 containing either pLAFR3, pAX24, or pLAF600 were analyzed by immunoblotting. Protein samples were heated for 10 min at 95°C before loading. After growth of strain DZQ40(pLAF600) on a plate, high-molecular-weight material was detected with the anti-$XcpQ_{aer}$ antiserum (Fig. 5), indicating the formation of the stable $XcpQ_{alc}$ multimeric complex. Such material was not detected for the other two strains. Some monomeric $XcpQ_{alc}$ was also detected. Interestingly, when strain DZQ40(pLAF600) was grown in liquid medium, the heat-stable $XcpQ_{alc}$ complex was not formed and only monomeric $XcpQ_{alc}$ was detected (Fig. 5). In contrast, when *P. alcaligenes* was grown in liquid medium, heat-stable $XcpQ_{alc}$ multimers were easily detected (Fig. 5).

In conclusion, the *xcp* genes from *P. alcaligenes* are expressed from pLAF600 in *P. aeruginosa* DZQ40 but heat-stable $XcpQ_{alc}$ multimers are formed efficiently in this strain only when the bacteria are grown on a plate. In contrast, in the natural host, heat-stable $XcpQ_{alc}$ multimers are also formed efficiently in liquid medium.

Complementation of *P. aeruginosa xcp* mutations by individual *P. alcaligenes xcp* genes or subsets of these genes. To study whether *P. alcaligenes* Xcp proteins can be combined with *P. aeruginosa* Xcp proteins to form a functional secreton, several subclones carrying *xcp* genes of *P. alcaligenes* were introduced in appropriate *P. aeruginosa xcp* mutants. Efficient complementation was obtained when pLAF600BH, carrying the *xcpR* to *-Z* operon of *P. alcaligenes*, was introduced in *xcpR* to *-Z* deletion mutant PAO1ΔRZ. This result was found both on plates (Fig. 6A) and in liquid medium (Fig. 6B and 7). Thus, XcpR-Z_{alc} formed a fully functional mixed secreton with $XcpPQ_{aer}$ in *P.*

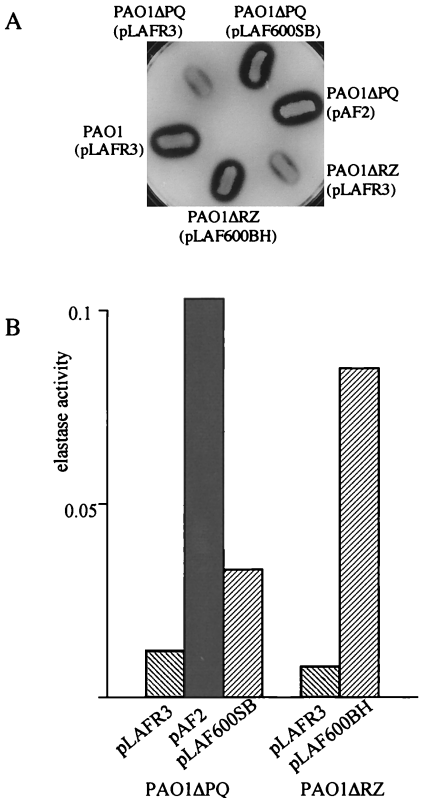

FIG. 6. Complementation of *P. aeruginosa xcpPQ* and *xcpR* to *-Z* deletion mutants by the *P. alcaligenes xcpPQ* and *xcpR* to *-Z* operons. Plasmids pLAF600SB and pLAF600BH carry the *P. alcaligenes xcpPQ* and *xcpR* to *-Z* operon, respectively. Plasmid pAF2 carries the *P. aeruginosa xcpPQ* operon. (A) Plate assay demonstrating protease activity. (B) Extracellular elastase activity. Samples were incubated with elastin-Congo red for 3 h. The activities are expressed as the amounts of liberated Congo red (OD_{495}) per OD_{600} unit.

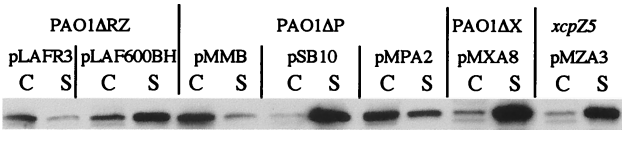

FIG. 7. Complementation of *P. aeruginosa xcp* mutations by *P. alcaligenes xcp* genes. Cellular (C) and supernatant (S) proteins corresponding to equal amounts of culture were analyzed by immunoblotting using an antiserum directed against elastase. The *xcpR* to *-Z* mutant PAO1ΔRZ contained either pLAFR3 or pLAF600BH carrying *P. alcaligenes xcpR* to *-Z*. The *xcpP* mutant PAO1ΔP contained either pMMB67HE (pMMB), pSB10 carrying *P. aeruginosa xcpP*, or pMPA2 carrying *P. alcaligenes xcpP*. Plasmids pMXA8 and pMZA3, carrying *P. alcaligenes xcpX* and *xcpZ*, respectively, were introduced into corresponding mutants PAO1ΔX and KS902-503 (*xcpZ5*).

TABLE 3. Heterologous complementation of *P. aeruginosa xcp* mutations by *P. alcaligenes xcp* genes

P. aeruginosa mutation	*P. alcaligenes xcp* gene(s)	Complementation[a] for strains grown:	
		On a plate	In liquid medium
Δ*xcpP* to -*Z*	*xcpP* to -*Z*	+	−
Δ*xcpPQ*	*xcpPQ*	+	−
Δ*xcpR* to -*Z*	*xcpR* to -*Z*	+	+
Δ*xcpP*	*xcpP*	−	−
Δ*xcpQ*	*xcpQ*	−	−
Δ*xcpRS*	*xcpR* to -*V*	+	+
Δ*xcpT*	*xcpT*	+	+
Δ*xcpX*	*xcpX*	+	+
xcpY51	*xcpY*	+	+
xcpZ5	*xcpZ*	+	+

[a] +, restoration of secretion; −, no restoration.

aeruginosa. The *xcpPQ* deletion of mutant strain PAO1ΔPQ was as efficiently complemented on plates with pLAF600SB as on plates with pAF2 (pLAF600SB and pAF2 contain the *xcpPQ* genes of *P. alcaligenes* and *P. aeruginosa*, respectively) (Fig. 6A). The proteolytic activity observed was caused by Xcp-dependent secretion since it was not observed when pLAF600SB was introduced in an *xcpR* mutant (results not shown). Thus, $XcpPQ_{alc}$ could form a functional secreton with $XcpR\text{-}Z_{aer}$ in *P. aeruginosa*. In liquid medium, however, the amount of extracellular elastase activity was much less with pLAF600SB than with pAF2, indicating only partial complementation under these growth conditions (Fig. 6B). Again, the lack of full complementation seemed to be related to inefficient formation of heat-stable $XcpQ_{alc}$ multimers in *P. aeruginosa*, since these multimers were hardly detected or not detected after growth of PAO1ΔPQ(pLAF600SB) in liquid medium but were easily detected after growth on plates (results not shown).

Further complementation experiments were performed with individual *P. alcaligenes xcp* genes, cloned under control of the *tac* promoter of pMMB67EH/HE (Table 3). With individual genes from the $xcpR\text{-}Z_{alc}$ operon, secretion was restored both on skim milk plates (results not shown) and in liquid medium (Fig. 7 and results not shown) in all cases tested. These results show that the Xcp proteins encoded by the *xcpR* to -*Z* operons of *P. aeruginosa* and *P. alcaligenes* can be combined into a functional secreton. However, introduction of *P. alcaligenes xcpP* and *xcpQ* in the corresponding *P. aeruginosa* mutants did not restore secretion, either on plates (results not shown) or in liquid medium (Fig. 7 and results not shown). Therefore, $XcpP_{alc}$ and $XcpQ_{alc}$ cannot replace $XcpP_{aer}$ and $XcpQ_{aer}$, respectively, except when they are expressed simultaneously in bacteria grown on plates (Table 3).

Species-specific stabilization of XcpP by XcpQ. The observation that *P. aeruginosa xcpP* and/or *xcpQ* deletions were only complemented by *P. alcaligenes xcpP* and *xcpQ* when these genes were expressed simultaneously indicates that XcpP and XcpQ interact in a species-specific way. Previously, an interaction between XcpP and XcpQ was suggested by the instability of $XcpP_{aer}$ in *P. aeruginosa xcpQ* mutant PAG2 (3). Here, we investigated whether $XcpQ_{alc}$ could stabilize $XcpP_{aer}$. Derivatives of strain PAG2 containing either pMMB67HE, pMB4 carrying *P. aeruginosa xcpQ*, or pCK28 carrying *P. alcaligenes xcpQ* were grown without IPTG, since the addition of IPTG was lethal for the strains containing pMB4 and pCK28. Immunoblotting showed that the levels of $XcpP_{aer}$ were strongly increased in the presence of $XcpQ_{aer}$ but only moderately increased in the presence of $XcpQ_{alc}$ (Fig. 8), indicating that $XcpQ_{alc}$ is much less efficient than $XcpQ_{aer}$ in stabilizing $XcpP_{aer}$. These results suggest that XcpP is stabilized by a species-specific interaction with XcpQ.

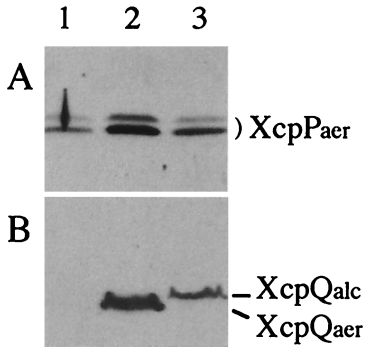

FIG. 8. Species-specific stabilization of XcpP by XcpQ. Equal amounts of total cell extracts of *xcpQ* deletion mutant PAG2 containing either pMMB67HE (lane 1), pMB4 carrying *P. aeruginosa xcpQ* (lane 2), or pCK28 carrying *P. alcaligenes xcpQ* (lane 3) were analyzed with antisera directed against $XcpP_{aer}$ (A) and $XcpQ_{aer}$ (B). Protein samples were heated for 10 min at 95°C and loaded on SDS-PAGE gels containing 11 (A) or 9% (B) acrylamide. Two different start codons in the *P. aeruginosa xcpP* gene are used as translation start sites, resulting in two distinct XcpP bands (A) (3). No heat-stable XcpQ complex, only monomeric XcpQ, was detected (B).

DISCUSSION

In spite of the conservation of the type II secretion system in many gram-negative bacteria, several studies indicate that the secretion of an exoprotein expressed in a heterologous host does not generally occur (10, 30, 45, 52). For example, very closely related *Erwinia* species *E. chrysanthemi* and *E. carotovora* cannot secrete the cellulases and pectate lyases of each other (30, 52). Such specific recognition suggests the presence of a secretion motif on the exoproteins that is recognized by one or more components of the Gsp machinery. Lindeberg et al. (41) proposed that OutC (GspC) and OutD (GspD) might be the gatekeepers involved in species-specific exoprotein recognition, since, except for OutC and OutD, each Out protein from *E. carotovora* could substitute for its counterpart from *E. chrysanthemi*. Similarly, except for PulC (GspC) and PulD (GspD), all Gsp components of *Klebsiella oxytoca* could be replaced by the corresponding proteins of *E. chrysanthemi* and *E. carotovora* (48). Furthermore, Shevchik et al. (55) reported that the Nterminus of OutD might indeed species-specifically bind the exoprotein substrate. However, a chimeric GspD protein, in which the N-terminal domain of *K. oxytoca* PulD was replaced by the corresponding domain of *E. chrysanthemi* OutD, supported pullulanase secretion via the *K. oxytoca* Gsp (Pul) system (26). The same study reported that the C-terminal domain of PulD cannot be replaced by its counterpart from *E. chrysanthemi*, OutD, without affecting pullulanase secretion. In contrast, Hardie et al. (29) reported that the C-terminal half of

E. chrysanthemi OutD could substitute for the C-terminal half of PulD. These contradictory results make it unclear whether and how the GspD homologs might be involved in species-specific recognition of the exoproteins.

Previously, we studied the species specificity of secretion between *P. aeruginosa* and *Pseudomonas putida* (10–12). However, despite the presence of an *xcp* gene cluster in *P. putida*, it is not clear whether it encodes a functional secreton, since no Xcp-dependent exoproteins were detected in this strain (12). In the present study, we analyzed the species specificity of secretion by *P. aeruginosa* and *P. alcaligenes*. Similarly as reported for *E. chrysanthemi* and *E. carotovora*, we observed that the individual XcpP (GspC) and XcpQ (GspD) proteins of *P. aeruginosa* could not be replaced by XcpP and XcpQ from *P. alcaligenes*, respectively. However, the lack of complementation, in this case, cannot be related to the species specificity of exoprotein recognition by XcpP and/or XcpQ. First, *P. aeruginosa* elastase and *P. alcaligenes* lipase were Xcp-dependently secreted when produced in heterologous hosts *P. alcaligenes* and *P. aeruginosa*, respectively. Second plate assays showed that introduction of a cosmid carrying the *xcpP* to *-Z* genes of *P. alcaligenes* in a *P. aeruginosa xcpP* to *-Z* deletion strain restored Xcp-dependent protein secretion. The failure of $XcpP_{alc}$ and $XcpQ_{alc}$ to complement the corresponding *P. aeruginosa* mutants may therefore be caused by species-specific interactions between these two proteins. Indeed, when *P. alcaligenes xcpP* and *xcpQ* were expressed simultaneously under the control of their native promoters, elastase secretion in a *P. aeruginosa xcpPQ* deletion mutant on skim milk plates was restored. Moreover, unlike $XcpQ_{aer}$, $XcpQ_{alc}$ failed to stabilize $XcpP_{aer}$.

The results of the complementation experiments also indicate that putative interactions of XcpPQ with other known Xcp proteins (XcpR to -Z) are not species specific, at least not between *P. aeruginosa* and *P. alcaligenes*. Indeed, $XcpPQ_{aer}$ and $XcpR\text{-}Z_{alc}$ formed a mixed secretion that supported efficient protein secretion in *P. aeruginosa*. Similarly, $XcpPQ_{alc}$ and $XcpR\text{-}Z_{aer}$ apparently formed a functional mixed secreton in *P. aeruginosa* when the bacteria were grown on plates. Interestingly, complementation of the *P. aeruginosa xcpPQ* mutant by the *P. alcaligenes xcpPQ* genes was not efficient when the bacteria were grown in liquid medium. This lack of complementation is again not due to the species specificity of the interactions of XcpPQ with other known Xcp proteins, since the same results were even found when the complete *P. alcaligenes xcpP* to *-Z* gene cluster was expressed in the *P. aeruginosa xcpP* to *-Z* deletion mutant. The inefficient complementation in liquid medium is not caused by inefficient recognition of the heterologous exoproteins by the secreton under these conditions, since the *P. alcaligenes* lipase was also not secreted via the Xcp_{alc} secreton in *P. aeruginosa* in liquid medium. Furthermore, *P. aeruginosa* elastase was secreted Xcp dependently by *P. alcaligenes* when grown in liquid medium. Therefore, these results suggest conditional defects in the assembly and/or functioning of the *P. alcaligenes* secreton in *P. aeruginosa*. Consistent with this idea, heat-stable multimers of $XcpQ_{alc}$ were only found after growth in conditions that supported secretion, whereas only monomers were found when the cells were grown in liquid medium. In conclusion, these data indicate that the assembly of the *P. alcaligenes* XcpQ complex, and possibly of the complete secretory apparatus, might involve interactions of $XcpQ_{alc}$ and/or $XcpP_{alc}$ with additional component(s) that are present in *P. alcaligenes* but lacking in *P. aeruginosa*. However, such a putative component(s) is apparently not essential during growth on a plate. Alternatively, such components might be present in *P. aeruginosa* but the interaction with $XcpP_{alc}$ and/or $XcpQ_{alc}$ might be too weak during growth in liquid medium, resulting in inefficient secreton assembly.

A protein possibly required for XcpQ assembly might be a putative PulS homolog. PulS is an outer membrane lipoprotein that protects the XcpQ homolog PulD of *K. oxytoca* from proteolytic degradation and that is required for insertion of PulD in the outer membrane (28, 29). Although PulD multimers were formed in the absence of PulS, they were composed of PulD degradation products (28). PulS homologs (OutS) have been identified in *E. chrysanthemi* (55) and *E. carotovora* (41). Analysis of the whole genome sequence (http://www.pseudomonas.com) did not reveal the existence of a *pulS* homolog in *P. aeruginosa*. Nevertheless, a functional homolog of PulS might be present in *P. alcaligenes* and could be required for efficient assembly of $XcpQ_{alc}$ in fast-growth conditions.

Recently, it was shown that two other Gsp proteins (probably) interact with the GspD secretin, i.e., XpsN (GspN) of *Xanthomonas campestris* (40) and OutB (GspB) of *E. chrysanthemi* (9). Genes encoding GspN have been identified in the *gsp* gene clusters of most GSP-containing bacteria, except *E. chrysanthemi, P. aeruginosa*, and *P. alcaligenes* (25, 40). Besides *E. chrysanthemi*, *Aeromonas hydrophila* (34), *E. carotovora* (41), and *K. oxytoca* (13) have been found to contain *gspB* genes. In *A. hydrophila*, the *gspB* (*exeB*) gene is clustered with the *gspA* (*exeA*) gene (34). Both the *exeA* and *exeB* gene products, which form a complex, are required for efficient type II secretion in this species (54). Genome sequence analysis did not reveal the presence of a *gspA*, *gspB*, or *gspN* gene in *P. aeruginosa*. Therefore, the assembly and/or functioning of the *P. alcaligenes* Xcp machinery in *P. aeruginosa* may have been affected by the absence of putative $GspA_{alc}$, $GspB_{alc}$, and $GspN_{alc}$ homologs.

Several studies indicate the involvement of an additional uncharacterized gene product(s) in type II secretion. For example, in addition to the characterized *gsp (out)* genes, an uncharacterized region of 4 kb upstream of *outS* appeared to be required for the reconstitution of the Gsp system of *E. chrysanthemi* in *E. coli* (41). This DNA segment might encode factors required for the assembly of the secretion machinery. Hamood et al. (27) described a pleiotropic *P. aeruginosa* secretion mutant that could not be complemented by *xcpA* or by the *xcpP* to *-Z* gene cluster, indicating the existence of an additional gene(s) required for secretion. Finally, Kagami et al. (35) reported the isolation of suppressors of an *xcpT* mutation in *P. aeruginosa*. One class of suppressor mutations mapped outside the *xcpP* to *-Z* gene cluster, again indicating that additional gene products may be involved in type II secretion. These uncharacterized gene products might be involved in the biogenesis of cell envelope components other than the secretion itself. It is likely that cell envelope processes, such as peptidoglycan or lipopolysaccharide (LPS) biosynthesis, affect the assembly and/or functioning of the secretion machinery. Consistently, *P. aeruginosa* strains with defective LPS were affected in the functioning of the Xcp secreton (44). Further-

more, LPS biosynthesis genes are required for type IV pilus biogenesis in *Vibrio cholerae*, a process that is related to type II protein secretion (33).

In conclusion, one or more of these additional gene products might be involved in *Pseudomonas* secreton assembly. These additional components might species specifically interact with XcpP and/or XcpQ, since these two components of *P. aeruginosa* could not efficiently be replaced by their homologs from *P. alcaligenes*. The construction of XcpP and XcpQ chimeric proteins is under way to identify the domains required for specific interaction between these two proteins and/or with the other, as yet unidentified gene products.

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