Mutual stabilization of the XcpZ and XcpY components of the secretory apparatus in *Pseudomonas aeruginosa*

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Protein secretion in Gram-negative bacteria is often dependent on the general secretory pathway (GSP). In *Pseudomonas aeruginosa*, this system requires at least 12 Xcp (Gsp) proteins, which are proposed to constitute a multiprotein complex localized in the bacterial envelope. Hitherto, little was known about the mutual interactions between Xcp proteins. In this study, mutants affected in the *xcpZ* gene encoding a bitopic inner-membrane protein were analysed to investigate the role of this protein in the architecture of the secretory machinery. The absence of XcpZ resulted in a decreased amount of XcpY. Reciprocally, XcpZ was not detectable in a *xcpY* mutant, demonstrating a mutual stabilization of these two proteins. These results strongly suggest that XcpZ and XcpY interact within the functional secretory apparatus.

Keywords: general secretory pathway, protein secretion, protein–protein interaction, protein stability, *Pseudomonas aeruginosa*

INTRODUCTION

Translocation of most of the Sec-dependent extracellular proteins across the outer membrane of Gram-negative bacteria occurs via the main terminal branch (MTB) of the general secretory pathway (GSP) (Pugsley, 1993). The MTB of the GSP machineries consists of a set of at least 12 specific proteins (Gsp proteins) and is widely disseminated in the bacterial species so far examined (Tomassen *et al.*, 1992; Lazdunski *et al.*, 1996).

The GSP has been shown to be the major pathway for the secretion of toxins and degradative enzymes in *Pseudomonas aeruginosa* (Filloux *et al.*, 1990), and in this bacterium the Gsp proteins are named Xcp. The XcpA protein (referred to as GspO in other Gram-negative bacteria) has been reported to be identical to the peptidase PilD, which catalyses maturation and methylation of the type IV pilins (Nunn & Lory, 1991; Strom *et al.*, 1993). Five other Gsp proteins, XcpT (GspG), -U (-H), -V (-I), -W (-J) and -X (-K) show homologies to PilA, the structural subunit of the type IV

pilus in *P. aeruginosa*. Since XcpT–K also require XcpA/PilD for their maturation, they have been referred to as pseudopilins (Bally *et al.*, 1992; Nunn & Lory, 1993; Bleves *et al.*, 1998). These pseudopilins are presumably present in both membranes, as judged by fractionation studies (Bally *et al.*, 1992; Pugsley & Possot, 1993). XcpQ (GspD) (Akrim *et al.*, 1993) is the sole protein of the machinery located in the outer membrane, whereas the other Xcp proteins, XcpP (GspC), -R (-E), -S (-F), -Y (-L), -Z (-M) and -A are integrated in, or associated with, the inner membrane (Reeves *et al.*, 1994; Sandkvist *et al.*, 1995; Bleves *et al.*, 1996; Thomas *et al.*, 1997).

Whereas the function of XcpA has clearly been demonstrated, little is known about the functions of the other individual Gsp proteins. Recently, it has been shown that the purified outer-membrane protein XcpQ has a ring-shaped multimeric structure with a central opening, through which the exoproteins probably reach the extracellular medium (Bitter *et al.*, 1998). In some cases, the membrane insertion of GspDs requires the chaperone activity of GspS (Hardie *et al.*, 1996), but GspS homologues have not yet been found in all systems described so far, including the *P. aeruginosa* system.

Except for *gspO*, the *gsp* genes of unrelated bacterial species are usually not interchangeable (de Groot *et al.*,

Abbreviations: GSP, general secretory pathway; MTB, main terminal branch.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Reference/source*
Strains		
<i>P. aeruginosa</i>		
PAO1	Prototroph, <i>chl</i> -2	B. Holloway ¹
PAO503	<i>met</i> -9011	B. Wretling ²
KS902/503	Isogenic <i>xcpZ</i> 5 mutant from PAO503	Wretling & Pavlovskis (1984)
KS911/503	Isogenic <i>xcpZ</i> 52 mutant from PAO503	Wretling & Pavlovskis (1984)
KS910/503	Isogenic <i>xcpY</i> 51 mutant from PAO503	Wretling & Pavlovskis (1984)
<i>E. coli</i>		
TG1	<i>supE</i> , <i>hsd</i> Δ <i>R</i> , <i>thi</i> Δ(<i>lac</i> - <i>proAB</i>) F'(<i>traD</i> 36 <i>proAB</i> ⁺ <i>laqI</i> ^a <i>lacZ</i> Δ <i>M</i> 15)	
Plasmids		
pBluescript	Ap ^R	Stratagene
pMMB67HE/EH	Ap ^R , RSF replicon (IncQ), <i>tac</i> promoter	Fürste <i>et al.</i> (1986)
pMMB190	Ap ^R , pMMB66EH, <i>tac</i> promoter, LacZα	Morales <i>et al.</i> (1991)
pLAFR3	Cosmid vector derived from pLAFR1, IncP1, Tc ^R	Friedman <i>et al.</i> (1982)
pUCBM20	Ap ^R	Boehringer
pYZ4	Km ^R , <i>lacUV5</i> p	Zhang & Broome-Smith (1990)
pPHO7	Ap ^R , <i>phoA</i> gene flanked by restriction sites	Gutierrez & Devedjian (1989)
pHP45ΩTc	Tc ^R , Ω interposon	Fellay <i>et al.</i> (1987)
pUEX383	Ap ^R , 3.9 kb <i>EcoRI</i> fragment with <i>xcpX</i> , - <i>Y</i> and - <i>Z</i> in pUC19	de Groot <i>et al.</i> (1991)
pSB22	1.8 kb <i>EcoRI</i> / <i>NotI</i> fragment of pUEX383 carrying the <i>xcpZ</i> gene in pUCBM20	This study
pSB12	1.8 kb <i>HindIII</i> / <i>EcoRI</i> fragment of pSB22 carrying the <i>xcpZ</i> gene in pMMB67HE	This study
pSB51	800 bp <i>EcoRI</i> / <i>HindIII</i> fragment encoding ss'-XcpZp in pMMB190	S. Bleves ³
pGB1	1.8 kb <i>HindIII</i> / <i>EcoRI</i> fragment of pSB22 (expressing XcpZ) in pYZ4	This study
pGB2	2.3-kb <i>SmaI</i> fragment of pHP45Ω inserted at the <i>ScaI</i> site of pGB1 (expressing XcpZmc')	This study
pGB3	1.2 kb <i>EcoRV</i> fragment of pGB2 in pMMB190	This study
pGB4	1.2 kb <i>KpnI</i> / <i>EcoRI</i> fragment of pGB3 in pMMB67HE	This study
pGB5	2.6 kb <i>SmaI</i> fragment of pPHO7 in the <i>ScaI</i> site of pGB1 (expressing XcpZmc'-PhoA)	This study
pGB6	3.2 kb <i>SalI</i> fragment of pGB5 in pMMB190	This study
pSB31	1.4 kb <i>SalI</i> / <i>SphI</i> fragment carrying the <i>xcpY</i> gene in pMMB67EH	This study
pSB72	800 bp <i>EcoRI</i> / <i>HindIII</i> fragment encoding ss'-XcpYp in pMMB190	S. Bleves
pMYS	700 bp <i>SalI</i> / <i>HindIII</i> fragment encoding XcpYc' in pMMB190	M. Bally ⁴
pMY'-PhoA	3.4 kb <i>PstI</i> fragment encoding XcpYcm'-PhoA on pMMB67HE	M. Bally
pLAFRY	1.6 kb <i>EcoRI</i> / <i>HindIII</i> fragment carrying <i>xcpY</i> in pLAFR3	M. Bally

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1991; Pugsley, 1996), suggesting highly specific interactions between the components of the GSP machineries. Yet, in the case of related species (Lindeberg *et al.*, 1996), or for Gsp proteins having a high percentage of identity (Francetic & Pugsley, 1996), exchangeability can be observed, resulting in the formation of hybrid machineries with variable efficiencies. Cross-linking experiments have pointed out that PulG of *Klebsiella oxytoca* is able to multimerize in *Escherichia coli* (Pugsley, 1996). Moreover, the Xcp homologue of PulG, XcpT, has been shown to interact with three other pseudopilins, XcpU, -V and -W (Lu *et al.*, 1997), and with XcpR (Kagami *et al.*, 1998). An alternative way to identify protein-protein interactions is to study the

stabilization of one protein by another. For example, Sandkvist *et al.* (1995) have shown that EpsE of *Vibrio cholerae* is associated with the cytoplasmic membrane and stabilized via interaction with EpsL, the Gsp homologue of *P. aeruginosa* XcpY. This approach has been extensively used in the case of membrane proteins which belong to complexes such as TonB/ExbB (Skare & Postle, 1991), SecE/SecY (Nishiyama *et al.*, 1992), the *Aeromonas hydrophila* GSP components ExeA/ExeB (Howard *et al.*, 1996) or the VirB T-complex transport machinery in *Agrobacterium tumefaciens* (Fernandez *et al.*, 1996).

Despite some recent advances, specific interactions

among most of the Xcp proteins have not yet been demonstrated. In this work, using *xcpZ* mutants, we investigated whether XcpZ stabilizes other Xcp proteins. Our findings support a model in which XcpZ and XcpY interact in the functional secretory apparatus in *P. aeruginosa*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains were grown in tryptone soy broth (Difco) at 37 °C under agitation and on tryptic soy agar plates containing skim milk (Difco).

DNA manipulations. All plasmid constructions were isolated from *E. coli* TG1 after transformation. Plasmids were transferred to *P. aeruginosa* by electroporation essentially as described by Smith & Iglewski (1989). Transformants were isolated on PIA (*Pseudomonas* isolation agar) plates containing 300 µg carbenicillin ml⁻¹ or 200 µg tetracycline ml⁻¹. *Pseudomonas* chromosomal DNA was prepared according to Marmur (1961).

Protein analysis. Cells were harvested at late-exponential phase, and proteins were precipitated with 10% TCA and washed twice with 90% acetone. The proteins were then solubilized and separated by SDS-PAGE as described by Laemmli (1970).

Immunoblot analysis. To compare the composition of the cell extracts, the protein contents of equivalent amounts of cells (corresponding to 1 ml culture with an OD₆₀₀ of 0.1) were loaded on polyacrylamide gels. Proteins, separated by SDS-PAGE on 10.5 or 15% (w/v) polyacrylamide gels, were transferred to nitrocellulose membranes using a semi-dry blot apparatus (Bio-Rad) and incubated with appropriately diluted antisera. Each experiment was performed twice. Either alkaline phosphatase- or peroxidase-conjugated goat anti-rabbit IgG (Immunotech or Sigma, respectively) were used as secondary antibody. Proteins were revealed by detection of alkaline phosphatase activity or by chemiluminescence (Pierce). The XcpZ rabbit antiserum was raised against a purified recombinant protein obtained by using the pEX fusion system, and XcpY antibodies were raised against a purified glutathione *S*-transferase (GST)-XcpY fusion protein. Semi-quantitative estimation of the relative amounts of proteins detected by chemiluminescence on autoradiography films was carried out by densitometric analysis using a Bio-Rad scanner.

Sequence of the *xcp* mutant genes and computer analysis. The *xcpY51* allele was amplified by PCR using chromosomal DNA from strain KS910/503 as a template and primers OYG1 (5' AAGAAGGACGAGCGATGAGT 3') and OYG2 (5' TGCGTCATCACCTTCATCTCA 3'). The PCR product was cloned into pUCBM20 and the sequence analysis carried out by ESGS Company (France). The *xcpZ5* and *xcpZ52* alleles were amplified by PCR using chromosomal DNA templates from strains KS902/503 and KS911/503, respectively, and primers OZG1 (5' GAGAGGATCCCCTGGTG-ATAGGAGGTT 3') and OZG2 (5' TATAGAATTCATCG-GCCTGTGCGCCACACGGT 3'). Direct sequencing on PCR products was carried out by the IBSM sequencing laboratory. Characteristics of predicted mutant proteins were analysed on the Expaty World Wide Web molecular biology server using the ProtParam tool program (Guruprasad *et al.*, 1990).

RESULTS AND DISCUSSION

The amount of XcpY is decreased in *xcpZ* mutants

Little is known about the mutual stabilization of components of the Xcp secretory machinery in *P. aeruginosa*, which we therefore decided to investigate. As a first step, to avoid any polar effect on the expression of other *xcp* genes, we focused on the influence of mutations in the last gene of the *xcp* operon, *xcpZ*. The effect associated with the lack of XcpZ was studied on four other Xcp proteins for which antibodies were available, i.e. XcpQ located in the outer membrane, XcpR peripherally associated with the inner membrane (M. Bally and others, unpublished), the pseudopilin XcpT and the bitopic cytoplasmic membrane protein XcpY (Bleves *et al.*, 1996). Two different *xcpZ* mutants were used in this study, KS902/503 (*xcpZ5*) and KS911/503 (*xcpZ52*) (Wretling & Pavlovskis, 1984). DNA sequencing (see Methods) revealed that the former mutation is a substitution of a thymine for a cytosine (C16T), introducing a stop codon near the 5' end of the gene. The second mutation is a similar substitution at position 379 in the DNA sequence, generating a stop codon in the DNA encoding the C-terminal periplasmic domain of the protein. In this case, the predicted product should be 5.2 kDa smaller than the wild-type protein. However, the mutant XcpZ protein was not detected in Western blot experiments (data not shown).

Cells from the parent strain PAO503 and the two isogenic *xcpZ* mutants were grown to late-exponential phase and their proteins were separated by SDS-PAGE. Interestingly, immunoblotting analysis showed that the amount of XcpY was decreased by 25–50% in the two mutants as compared to the parent strain (Figs 1a, 2b). In contrast, the levels of XcpR (Fig. 1b), XcpQ (data not shown) and XcpT (data not shown) were not affected.

Fig. 1. Amount of XcpY and XcpR in *xcpZ* mutants. Cell extracts from *P. aeruginosa* wild-type (PAO503) and isogenic mutants (*xcpZ5* and *xcpZ52*) were analysed by Western blotting using anti-XcpY (a) or anti-XcpR (b) antibodies. Immunodetection was carried out by chemiluminescence. Proteins cross-reacting non-specifically with anti-XcpY and anti-XcpR antibodies are indicated by arrows on the right and were used as quantitative markers. The amount of XcpY protein is expressed as a percentage relative to that of the wild-type strain.

Fig. 2. Amount of XcpY in *xcpZ* mutants complemented by the wild-type *xcpZ* gene. Plasmid pSB12 carrying the wild-type *xcpZ* gene or control vector pMMB67HE were introduced into the wild-type PAO503 and into the *xcpZ5* and *xcpZ52* mutants. Proteins separated by SDS-PAGE (10.5% acrylamide) were analysed by Western blotting using XcpZ (a) and XcpY (b) antibodies. Immunodetection was carried out by chemiluminescence. The amount of XcpY and XcpZ proteins is expressed as a percentage relative to that of the wild-type strain not harbouring pSB12.

Moreover, this decreased level of XcpY appeared not to be due to an increased level of proteases in the periplasm caused by the secretion defect in the *xcpZ* mutants since it was not significantly affected in other *xcp* mutants, such as an *xcpQ* mutant (data not shown). These results suggest that XcpZ stabilizes XcpY.

xcpZ expressed *in trans* in *xcpZ* mutants restores XcpY stability

To check whether the reduced amounts of XcpY in the *xcpZ* mutants were directly related to the absence of XcpZ, the wild-type *xcpZ* gene was expressed *in trans* in the two *xcpZ* mutants (Fig. 2a). In these strains, the amount of XcpY was restored to the wild-type level (Fig. 2b). This result demonstrates that the amount of XcpY is indeed dependent on the presence of XcpZ. The *xcpY* gene is located in a large operon (*xcpR* to *-Z*) and it is unlikely that the decreased level of XcpY is due to a feedback inhibition of its synthesis in the *xcpZ* mutants since other gene products encoded by this operon were not significantly affected. Moreover, we compared the activity of an *xcpR-lacZ* transcriptional fusion (Chapon-Hervé *et al.*, 1997) in the *xcpZ* mutant and in the wild-type strain. No significant difference could be detected at the level of β -galactosidase activity in all strains tested (data not shown). Concomitant with the stabilization of XcpY, the defect of the *xcpZ* mutants in elastase secretion was complemented by the introduction of the plasmid harbouring *xcpZ* (pSB12), showing that the function of the Xcp machinery was restored in both mutants (data not shown).

XcpY is reciprocally required for the stability of XcpZ

To further establish a possible interaction between XcpZ and XcpY, it was relevant to question whether XcpY could reciprocally stabilize XcpZ. The mutant

Fig. 3. Effect of XcpY on XcpZ stability. Western blot analysis of PAO503 (wild-type) and *xcpY51* isogenic mutant cell extracts. Immunodetection was performed by chemiluminescence with anti-XcpZ (a) or anti-XcpY (b) as primary antibodies. The wild-type *xcpY* gene carried on pSB31 was introduced into *xcpY51* strain and expression of the gene induced by addition of 2 mM IPTG where indicated. The amount of XcpY and XcpZ proteins is expressed as a percentage relative to that of the wild-type strain not harbouring pSB31.

KS910/503 (*xcpY51*) provided an opportunity for studying such a relation. DNA sequencing revealed that this mutation *xcpY51* is an insertion of 7 nucleotides, GCGCAGA, between nucleotide residues 809 and 810 of *xcpY* in the region encoding the C-terminal periplasmic domain of the protein. Computing parameters (Guruprasad *et al.*, 1990) predicted an unstable product, 1.25 kDa larger than the wild-type protein and with a basic pI of 11.26 (compared to pI 5.22 for the wild-type protein). No mutant *xcpY* gene product was detected after SDS-PAGE and Western blot analysis of extracts from the *xcpY51* mutant (Fig. 3b), possibly because it is unstable and proteolytically degraded. Interestingly, XcpZ could not be detected in the *xcpY* mutant, showing that XcpY and XcpZ stabilities are tightly related (Fig. 3a). However, it should be pointed out that in the *xcpZ* mutants, XcpY was still present, but in reduced amounts, whilst no XcpZ was detected at all in the *xcpY* mutant. These results could be explained by a greater instability of XcpZ in the *xcpY* mutant compared to that of XcpY in *xcpZ* mutants. Since the GspE and GspL homologues of *V. cholerae* have been shown to interact, XcpY could partly be stabilized via interaction with XcpR, even if XcpZ is not present. Introduction of pSB31 carrying *xcpY* in the *xcpY51* mutant resulted in the recovery of a detectable level of XcpZ (Fig. 3a). It should be stressed that the optimal recovery of XcpZ was obtained when *xcpY* was not induced from the *tac* promoter. In these conditions, the amount of XcpY was similar to that obtained from the chromosomal *xcpY* copy (Fig. 3b). Upon induction with IPTG, high expression of XcpY resulted in its partial degradation (Fig. 3b) and in a diminished amount of XcpZ, (reduced to 66 % of the control value) (Fig. 3a). Corroborating these data, IPTG induction of *xcpY* expression prevented full complementation of the

Fig. 4. Amounts of XcpY and XcpZ produced in *E. coli*. Western blot analysis of TG1 cell extracts containing *xcpY* (pLAFRY), *xcpZ* (pSB12) or both. Immunodetection was performed by chemiluminescence with anti-XcpZ (a) or anti-XcpY (b) as primary antibodies. To prevent production of too large an amount of proteins, gene expression was not induced with IPTG. The amount of XcpY and XcpZ proteins is expressed as a percentage relative to that of the TG1 strain containing both plasmids.

xcpY51 mutant as concluded from the size of the haloes around colonies on skim-milk plates, which is a measure of the extracellular proteolytic activity (data not shown). The high production level under these conditions may prevent membrane insertion of XcpY, resulting in aggregation and proteolysis of the protein in the cytosol. Moreover, the results obtained emphasize the fact that the wild-type stoichiometry between the two proteins is important for the assembly of a functional secretory machinery. Whereas the mutual stabilization of XcpZ and XcpY strongly suggests an interaction between these proteins, attempts to directly demonstrate such an interaction were carried out by chemical cross-linking or co-immunoprecipitation using XcpY antibodies. However, in our hands, no physical interaction between these proteins could be detected, suggesting that the interaction between XcpZ and XcpY might be a transient event, or that the proteins associate with a low affinity.

Mutual stabilization of XcpY and XcpZ in *E. coli*

To demonstrate that the variation in the levels of XcpY and XcpZ reflects a direct interaction between the two components, the corresponding genes were expressed, individually or together, in *E. coli* and in the absence of all the other *xcp* genes. The plasmids carrying *xcpY* (pLAFRY) and *xcpZ* (pSB12) were transformed or co-transformed in *E. coli* TG1. Cell extracts from the different strains were then analysed by SDS-PAGE and Western blotting for the presence of the XcpY and XcpZ proteins. It appears that the coexpression resulted in an increased level of each protein as compared to the level obtained when each gene was expressed individually (Fig. 4). This result is strongly in favour of a direct interaction between XcpY and XcpZ, resulting in an increased stability of these proteins within the complex they form.

Fig. 5. (a) Amount of XcpY in the presence of various domains of XcpZ. The periplasmic domain of XcpZ was expressed as a truncated protein (ss-XcpZp) from pSB51, whilst the membrane and cytoplasmic domains of XcpZ (XcpZmc') were expressed as a truncated protein from pGB4 in the *P. aeruginosa* *xcpZ5* mutant. XcpY was analysed by Western blotting and the protein immunodetected by chemiluminescence. The amount of XcpY is expressed as a percentage relative to that of the wild-type strain. (b) Influence of various domains of XcpY on the amount of XcpZ. Plasmids carrying genes encoding ss-XcpYp (pSB72), XcpYc' (pMY5), XcpYcm'-PhoA (pMY'-PhoA) and XcpY (pSB31) were introduced into the *xcpY51* mutant strain. Expression of *xcpY* and derivatives was induced with 2 mM IPTG. Cell extracts were analysed by Western blotting and immunodetection was performed by chemiluminescence using anti-XcpZ antisera.

Separate protein domains of XcpZ or XcpY are not able to restore the stability of full-size XcpY or XcpZ, respectively

To investigate whether XcpY could be stabilized by the periplasmic domain of XcpZ alone, plasmid pSB51 encoding a mutant XcpZ protein (ss-XcpZp), in which the cytoplasmic domain and the membrane anchor of XcpZ are replaced by the signal peptide of the *P. aeruginosa* elastase (LasB), was introduced in the *xcpZ5* mutant strain. The truncated XcpZp protein could clearly be detected by immunoblotting (data not shown). However, expression of the periplasmic domain of XcpZ (XcpZp) did not stabilize XcpY (Fig. 5a). The influence of membrane and cytoplasmic domains of XcpZ was also investigated. For this purpose, two constructs were engineered. The product of one construct, generated by the insertion of a stop codon in *xcpZ* (pGB4), could not be detected (data not shown) and did not stabilize XcpY (Fig. 5a). The other construct, pGB6 encoding a *xcpZ'*-*phoA* gene fusion, resulted in the synthesis of a protein of the expected size (54.6 kDa) which was revealed on immunoblots with antiserum directed against PhoA. As for pGB4, the presence of pGB6 did not stabilize XcpY (data not shown).

The influence of the XcpY domains on XcpZ was

Fig. 6. Effect of overexpression of XcpZ on the negative dominant effect caused by XcpY overproduction. Proteolytic activity was analysed on a skim-milk plate with *P. aeruginosa* containing the vectors pMMB67HE and pLAFR3 (a), plasmids expressing either *xcpZ* (pSB12) (b) or *xcpY* (pLAFRY) (c) or both simultaneously (d). The plate contained 500 µg carbenicillin ml⁻¹, 200 µg tetracycline ml⁻¹ and 2 mM IPTG.

similarly studied. Three constructs were used, one encoding the C-terminal periplasmic domain of XcpY fused to the LasB signal peptide (ss'-XcpYp encoded on pSB72), one encoding the N-terminal cytoplasmic domain of XcpY (XcpYc' encoded on pMYS), and one encoding the N-terminal domain including the trans-membrane segment fused to PhoA (XcpYcm'-PhoA encoded on pMY'-PhoA). Even though each of the proteins could be visualized by immunoblotting (data not shown), neither of them was sufficient to restore the wild-type level of XcpZ (Fig. 5b).

Thus, in this way, we could not identify a particular domain of either XcpZ or XcpY that is responsible for the stabilizing effect. Rather, these results suggest that expression of the truncated forms could result in misfolded protein domains, which cannot properly interact, and therefore that the entire proteins might be necessary to promote a mutual stabilizing effect.

Interference associated with XcpY overexpression is relieved upon co-overexpression of XcpZ

As already mentioned, the stoichiometry of the Xcp components appears to be an important parameter for the proper functioning of the secretory machinery. Overexpression of XcpY from plasmid pLAFRY in the wild-type *P. aeruginosa* strain PAO1 had a dominant negative effect on protein secretion, referred to as interference, as revealed by the reduced proteolytic activity on skim-milk plates (Fig. 6). Since the data presented above suggest an interaction between XcpZ and XcpY, the interference due to XcpY overexpression could possibly be alleviated by co-overexpression of

XcpZ. Indeed PAO1 cells containing pSB12 (*xcpZ*) in addition to pLAFRY (*xcpY*) developed wild-type haloes on skim-milk plates (Fig. 6). Immunoblotting revealed that the level of XcpY produced in both cases was similar, indicating that the suppression of interference was due to XcpZ overexpression (data not shown).

Sandkvist *et al.* (1995) have shown that EpsE and EpsL from *V. cholerae* interact with each other, and it appears that XcpR and XcpY behave similarly within the Xcp system (M. Bally and others, unpublished). The interference phenomenon observed when XcpY is over-produced could be explained by the titration of XcpR with an excess of XcpY in areas of the membrane lacking an additional partner essential for the assembly of a functional Xcp machinery. On the basis of the data presented here, one could speculate that XcpZ is this additional partner. XcpZ would dock XcpY or the XcpY/XcpR complex to an appropriate membrane site for further assembly of the secretory machinery.

In conclusion, the data presented here argue in favour of a specific interaction between two proteins of a GSP secretory system, XcpZ and XcpY, which is an important addition to our understanding of the architecture of this machinery. The method used to show the interdependence between both proteins is very useful and will be further exploited when non-polar mutants in all *xcp* genes and antisera against all Xcp proteins become available.

ACKNOWLEDGEMENTS

The authors would like to thank Marc Bally for sharing unpublished data, and for giving plasmids pMYS and pLAFRY, and XcpZ and XcpR antisera; Wilbert Bitter for providing XcpQ antisera; Paul Sauve for oligonucleotide synthesis; Olivier Uderso for preparation of all media and buffers; and Jeanine Busutil for DNA sequencing. Sophie Bleves is supported by the Ministry of Research and Technology and the whole work was partly supported by the French cystic fibrosis foundation, AFLM (Association Française de Lutte contre la Mucoviscidose) and by the Biotech Framework IV grant number BI04 CT960119 from the European Union to Cell Factories Network.

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Received 19 March 1998; revised 30 July 1998; accepted 7 September 1998.