XphA/XqhA, a Novel GspCD Subunit for Type II Secretion in Pseudomonas aeruginosa

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The opportunistic human pathogen bacterium Pseudomonas aeruginosa secretes various exoproteins in its surrounding environment. Protein secretion involves different secretory systems, including the type II secretion system, or T2SS, that is one of the most efficient secretory pathways of P. aeruginosa. There are two T2SS in this bacterium, the quorum-sensing-regulated Xcp system and the Hxc system, which is only present under phosphate-limiting conditions. Like T2SS of other bacteria, the Xcp T2SS is species specific, and this specificity mainly involves two proteins, XcpP (GspC family) and the secretin XcpQ (GspD family), which are the gatekeepers of the system. Interestingly, an orphan secretin, XqhA, was previously reported as being able to functionally replace the XcpQ secretin. In this study, we identified another gene, which we named xphA (xcpP homologue A), which is located next to xqhA. We showed that deletion of the xphA gene in an xcpP mutant caused the disappearance of the residual secretion observed in this mutant strain, indicating that the protein XphA plays a role in the secretion process. Our results also revealed that complementation of an xcpP/xcpQ mutant can be obtained with the gene couple xphA/xqhA. The XphA and XqhA proteins (the P,Q subunit) could thus form, together with XcpR-Z, a functional hybrid T2SS. A two-dimensional polyacrylamide gel electrophoresis analysis showed that except for the aminopeptidase PaAP, for which secretion is not restored by the P,Q subunit in the xcpP/xcpQ deletion mutant, each major Xcp-dependent exoprotein is secreted by the new hybrid machinery. Our work supports the idea that components of the GspC/GspD families, such as XphA/XqhA or XcpP/XcpQ, are assembled as a specific tandem within the T2SS. Each of these pairs may thus confer a different level of secretion specificity, as is the case with respect to PaAP. Finally, using a chromosomal xphA-xqhA fusion, we showed that the xphA-xqhA genes are transcribed from an early stage of bacterial growth. We thus suggest that the P,Q subunit might be involved in the secretion process at a different growth stage than XcpP/XcpQ.

Pseudomonas aeruginosa is an opportunistic human pathogen bacterium which secretes a wide variety of virulence factors, including hydrolytic enzymes, into its surrounding environment. Secretion of these exoproteins requires different secretion systems, defined as type I, II, III, and V (16) and recently type VI (25), showing the high secretory diversity of this bacterium. To date, type IV is the only secretory system not identified in P. aeruginosa. The type II secretion system (T2SS) is conserved and widespread among bacterial species (13). This system, which is used by exoproteins bearing a signal peptide (Sec or Tat signature), is thought to be organized as a multiprotein complex spanning the bacterial envelope (12, 13, 27, 29). In P. aeruginosa, two independent T2SS named Xcp (or secreton) and Hxc (2) coexist. The xcp genes are organized in two divergent operons containing, respectively, xcpPQ and xcpRZ genes, while the genetic organization of the hxc genes is quite different (2, 13). The two machineries consist of 11 proteins named, respectively, XcpP to -Z (13) and HxcP to -Z (2) and require the presence of the peptidase XcpA/PihD, which is also involved in the maturation of PilA, the structural unit of type IV pili (26). The Xcp machinery is involved in the secretion of some of the major hydrolytic enzymes produced by P. aeruginosa, such as elastase (LasB), exotoxin A, or phospholipase C (13). Expression of the xcp genes has been shown to be under the positive control of quorum sensing (8, 35). In contrast, the Hxc system is functional only under phosphate limitation. This phosphate-regulated system was previously described to mainly secrete one enzyme, the low-molecular-weight alkaline phosphatase LapA (2).

Although T2SS are generally conserved, heterologous secretion is mostly species dependent, and components of the machinery are not systematically exchangeable between distant organisms (13). Moreover, T2SS components homologous to XcpP (GspC family) and to the secretin XcpQ (GspD family) have been suggested to be the gatekeepers of the system and to confer specificity for substrate recognition (6, 22).

Secretins are organized as multimers of 12 to 14 subunits and are the only outer membrane components of the T2SS. They form a ring-shaped structure with a central cavity, the diameter of which varies between 50 and 90 Å according to the species and constitutes the extrusion channel of the system. Secretins are also described as bipartite proteins which consist of a C-terminal domain (or homology domain) embedded in the outer membrane and conserved in all the members of the secretin family and an N-terminal domain more species specific which extends into the periplasm (3). In Erwinia chrysanthemoides, it has been shown that secretins are organized as multimers of 12 to 14 subunits and that the extrusion channel diameter is dependent on the species.
them, this domain was shown to interact with a T2SS-secreted enzyme (the pacte lyase PelIB), suggesting that it could be the determinant of the species specificity (32). In other respects, XcpP, the partner of the secretin XcpQ, is a bitopic inner membrane protein with a large periplasmic domain (4). XcpP presents some characteristics different from its homologues of other species. It was shown to migrate as two bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in agreement with the two initiation codons predicted by in silico analysis, and it contains a coiled-coil-interacting domain in contrast to the PDZ domain present in most of the XcpP homologues (5). Coiled-coil and PDZ domains exhibit the same function (protein-protein interaction) but are structurally different. Furthermore, using hybrid proteins obtained by domain swapping between XcpP and its E. chrysanthemi homologue, OutC, we identified a 35-residue region localized in the periplasmic domain of the protein which could be involved in species specificity (15). More recently, we reported that specificity could also involve the interaction of the C-terminal domain of XcpP with XcpQ and that, as previously suggested by Bleves et al. (5), such a specific interaction could promote a fine-tuning control of the secretion opening (28). These observations suggest that rather than involving one T2SS component, species specificity could depend at least on effective interactions occurring between XcpP and XcpQ via specific domains. T2SS components cannot be exchanged in distant organisms. However, in related species such as P. aeruginosa and Pseudomonas alcaligenes, although xcpP and xcpQ genes cannot be exchanged individually, they can restore the functionality of the Xcp system when they are exchanged pairwise (9). This observation is in agreement with a species-specific interaction between XcpP and XcpQ.

Besides the organized multiprotein Xcp and Hxc secretion systems, it was found that although not required for efficient secretion in wild-type P. aeruginosa, an individual protein, XqhaA, encoded by a gene isolated on the P. aeruginosa genome and highly homologous to the xcpQ gene was responsible for the residual secretion observed in a mutant of the P. aeruginosa PAK strain deleted of the xcpQ gene (23). XqhaA, which belongs to the secretin family, was also shown to require Xcp components to be functional and to recognize the exoproteins of the Xcp system. The presence in P. aeruginosa of a secretin not directly associated with the Xcp system but able to independently associate with the Xcp components and to restore secretion in an xcpQ-deleted strain can argue against the hypothesis of XcpP/XcpQ pairwise specificity. However, it should be pointed out that in the study reported by Martinez et al. (23) the xqhaA gene was part of a 7.5-kb DNA fragment that most probably included other genes.

In this study, we showed that upstream and adjacent to the xqhaA gene, an xcpP homologue that we have annotated in the P. aeruginosa PAO1 genome as xphA (for xcpP homologue; PAI867; www.pseudomonas.com) could clearly be identified. Such a genetic organization, reminiscent of xcpP and XcpQ organized into a single operon, suggests that similarly to XcpP and XcpQ, the XphA and XqhaA proteins could constitute a specific functional GspCD secretory unit involved in protein secretion. We further investigated the physiological role played by these proteins in the secretion process. Our results support the idea that XphA and XqhaA can associate with the XcpR-Z proteins of the classical T2SS of P. aeruginosa to constitute a hybrid secretion machinery with its own specificity.
nesence using specific horseradish peroxidase-conjugated secondary antibodies (SuperSignal West Pico luminol; Pierce).

Proteolytic activity assays on plates. Protease activities were assayed by grow-
ing cells on tryptic soy agar (TSA) containing skim milk incubated at 37°C.

RESULTS AND DISCUSSION

In silico analysis of XphA and XqhA. In silico analyses showed that the protein encoded by the PA1867 gene found in the PAO1 genome next to the previously identified xphA gene has significant similarities with XcpP. We named the gene PA1867 xphA for xcpP homologue. However, the putative xphA gene product is predicted to present some differences compared to its homologue XcpP. BlastP analysis revealed 36% identity (56/154 residues aligned) and 46% homology (72/154) between the two protein homologues. Sequence alignment using Clustal W (http://www.ebi.ac.uk/clustalw/) or T-COFFEE (http://www.ch.embnet.org/software/TCoffee.html) showed that XphA lacks a C-terminal extension present in XcpP (Fig. 1). XphA has a smaller size (175 amino acid residues versus 235), a higher pI (10.8 versus 5.46), and four cystein residues compared to XcpP. The shorter size of XphA might be explained by the absence of a coiled-coil domain (ch.EMBnet.org), which is characteristic of XcpP or of a PDZ domain present in most of the XcpP homologues in other species. These domains are usually found at the C terminus of XcpP or its homologues. Since XphA is predicted to be an inner membrane protein, we used the DAS Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS/) to determine the transmembrane domain of the protein. A typical transmembrane domain was predicted between residues 32 and 46 of XphA, at a position rather similar to that found for XcpP (residues 34 to 48). One of the interesting features of XcpP is that the gene which encodes this protein contains two initiation codons, giving two products of different molecular weights and which migrate as a doublet in SDS-PAGE (5). Cloning and expression of the two forms of the xcpP gene led to products that behave similarly in the secretion process and that do not appear to play a different physiological role (5). Analysis of the xphA DNA sequence revealed that, as for the xcpP gene, the xphA gene could encode two potential products (Fig. 1). The first one starts from the first base pair and stops at the end of the sequence (bp 528), giving a 175-amino-acid product, while the second might extend from bp 37 of the DNA sequence to bp 528 (163-amino-acid residue) (ATGpr server at www.hri.co.jp). BlastP searches established that XqhA and XcpQ present 63% identity (387/605 aligned residues) and 80% homology

### TABLE 1. Bacterial strains and plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
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<th>Reference or source</th>
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<td>TG1</td>
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</tr>
<tr>
<td>1046</td>
<td>met supE hsdS recA</td>
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<td><strong>Pseudomonas aeruginosa</strong> strains</td>
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<td>pKNΔxPΔxQ</td>
<td>Mutator plasmid for xphA-xqhA deletion</td>
<td>This work</td>
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<td>Cosmid derivative of pLAFR1, IncP1, Tc’</td>
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<td>pLAFR3 bearing all the xcp genes</td>
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<td>pMMB67HE bearing the xphA gene from the gateway system with V5 and His tags</td>
<td>G. Ball</td>
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<td>Mini CTX-pPΔxLacZ</td>
<td>Mini CTX-lacZ bearing a transcriptional xphA-lacZ fusion</td>
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**FIG. 1.** Sequence alignment of XcpP and XphA. The schematic representation of the proteins was drawn from sequence alignment using the Clustal W program. Numbers correspond to the amino acid position in the sequence. Arrows indicate the starts of the protein sequences, TM (gray), transmembrane domain; CC (black), coiled-coil domain.
and that it could contribute to the secretion process.

In contrast to the xcpP mutant (23), a slight hydrolysis halo corresponding to XphA protein has never been described in P. aeruginosa (data not shown). Furthermore, as for XphA and XcpP, the pl of XphA is also predicted to be higher than that of XcpQ (7.3 compared to 5.78).

Therefore, XphA and XphQ present some differences compared to their respective homologues, which could have a physiological significance and be related to peculiar functions played by these proteins. On the one hand, sequence alignment of XcpP and XphA showed the presence of a C-terminal extension in XphA which is absent in XcpQ (data not shown). Furthermore, as for XphA and XcpP, the pl of XphA is also predicted to be higher than that of XcpQ (7.3 compared to 5.78).

The secretion process of wild-type P. aeruginosa was analyzed by immunoblotting, probed either with V5 (lane 1) or penta-His (lane 2) antisera, and revealed by chemiluminescence using horseradish peroxidase-conjugated antibodies (Pierce). The apparent molecular mass of XphA was estimated from the position of protein standards (lane 3), from the top to the bottom, 97, 66, 45, 30, 20, and 14.4 kDa. IMAC purification of XphA (lanes 4 to 6) was carried out from protein extracts obtained from E. coli BL21 bearing pMMB67D-1867 as indicated in Materials and Methods. Lane 4, DM-soluble proteins; lane 5, flowthrough fraction; lane 6, purified fraction eluted with imidazole. Proteins from lanes 3 to 6 were stained with Imperial protein stain (Pierce).

V5-tagged version of the XphA protein was cloned from a gateway library (20) in the expression vector pMMB67HE, giving pMMB67D-1867. Gene expression was carried out in an xcpP deletion mutant of P. aeruginosa. Interestingly, immunoblotting experiments using a V5 antiserum showed for the first time that, like the xcpP gene, xphA was expressed in P. aeruginosa as a protein doublet (XphA\(_{\text{V5-His}}\)), exhibiting a molecular mass in SDS-PAGE close to 25 kDa (Fig. 3, lane 1). The xphA gene was also expressed in the E. coli BL21(DE3) strain for purification attempts. As depicted on the immunoblot probed with penta-His antiserum (Fig. 3, lane 2), XphA was mainly recovered in the elution fraction in the purification lane 6. Detergent screening for membrane solubilization showed that DM was found to be more efficient than Triton X-100 or N-octyl-β-D-glucopyranoside for XphA solubilization. DM-soluble proteins were purified on nitrocellulose acid-Ni\(^{2+}\) magnetic agarose beads, and proteins were analyzed by SDS-PAGE and Western blotting (Fig. 3, lanes 4 to 6). Coomassie blue staining revealed that XphA was mainly recovered in the elution fraction in the presence of imidazole (Fig. 3, lane 6). As expected, purified XphA was shown to migrate like its homologue XcpP as a protein doublet corresponding to proteins of close molecular mass (around 25 kDa).

A characteristic was confirmed by immunoblotting experiments with the purified fraction (data not shown).

**FIG. 2.** Functionality of XphA in P. aeruginosa. Wild-type and mutant strains deleted of the xcpP gene or of both the xcpP and the xphA genes were streaked on TSA-skim milk plates to assay secreted proteolytic activities. ΔP, xcpP-deleted mutant; ΔPP\(_A\), mutant deleted of both xcpP and xphA genes.

**FIG. 3.** Characteristics of the XphA protein. The xphA gene cloned on plasmid pMMB67D-1867 was expressed in an xcpP mutant of P. aeruginosa (lane 1) or in E. coli BL21(DE3) (lane 2). Proteins were analyzed by immunoblotting, probed either with V5 (lane 1) or penta-His (lane 2) antisera, and revealed by chemiluminescence using horse-radish peroxidase-conjugated antibodies (Pierce). The apparent molecular mass of XphA was estimated from the position of protein standards (lane 3), from the top to the bottom, 97, 66, 45, 30, 20, and 14.4 kDa. IMAC purification of XphA (lanes 4 to 6) was carried out from protein extracts obtained from E. coli BL21 bearing pMMB67D-1867 as indicated in Materials and Methods. Lane 4, DM-soluble proteins; lane 5, flowthrough fraction; lane 6, purified fraction eluted with imidazole. Proteins from lanes 3 to 6 were stained with Imperial protein stain (Pierce).
sated by homologous components such as XphA and XqhA. Indeed, in a quadruple mutant deleted of xcpP, xcpQ, xphA, and xqhA genes, no proteolysis halo was observed on the plate (Fig. 4A, lane 3), while LasB was found to have accumulated intracellularly (Fig. 4B, lane 3). These results indicated that XphA and XqhA are responsible for the residual secretion observed in the xcpP/xcpQ double mutant (Fig. 4, lane 2). GspC and GspD components of the T2SS are known to require homologues, XphA and XqhA can reconstitute in association with XcpR-Z as additional secretion components to constitute a functional hybrid T2SS secretion apparatus.

**Substrate specificity of the P_{AQA} secretion subunit.** The P_{AQA} secretion subunit appeared to work in the Xcp secretome, albeit less efficiently than XcpPO regarding the diversity of substrates secreted by this T2SS. Thus, we investigated the possibility for the P_{AQA} subunit to have a specific set of substrates by comparing the secretomes of the different strains used in this work. Extracellular proteins secreted during growth were separated by two-dimensional (2D)-PAGE and identified after MALDI-TOF mass spectrometry. Protein spots 1 to 5 (Fig. 6A) were reproducibly observed in the parental strain PAO1 grown under our standard culture conditions and were identified by mass spectrometry as the aminopeptidase PaAP (PA2939) for spot 1 (7), the alkaline protease AprA (PA1249) for spot 2, the chitin binding protein CbpD (PA0852) for spot 3, the elastase LasB (PA3724) for spot 4, and the protease PrpL (PA4175) for spot 5. Except for AprA, which is secreted by the T1SS, all the other proteins identified are suggested to be T2SS substrates. The secretome of the quadruple mutant Δ POP_{AQA} showed a drastic decrease of the four T2SS-dependent proteins, PaAP, CbpD, LasB, and PrpL, showing as expected an alteration of secretion via T2SS (Fig. 6A and C). Overexpression of the xphA and xqhA genes in this mutant strain restored at least partially the secretion of three out of the four T2SS-dependent exoproteins that were affected for secretion, confirming that although apparently less efficient than their respective Xcp homologues, XphA and XqhA can reconstitute in association with XcpR-Z a functional hybrid T2SS (Fig. 6C and D). Interestingly, the aminopeptidase PaAP (spot 1) was not recovered after complementation of the xphA/xqhA deletion under the growth conditions tested, suggesting that its secretion requires a higher degree of specificity than for the other T2SS substrates (Fig. 6B and D). These results were confirmed by SDS-PAGE and immunoblotting of cellular and extracellular protein extracts probed with PaAP antiserum. PaAP was only detected in the cell fraction of the quadruple mutant bearing either the empty vector or expressing xphA/xqhA, showing that the protein was normally produced but not secreted (data not shown). Control experiments using the Δ POP_{AQA} mutant

**FIG. 5.** Effect of xcpRZ deletion on the secretory activity of P_{AQA}. The secretory activity of the P_{AQA} subunit was studied in a mutant strain deleted of the xcpRZ operon (ΔRZ). (A) Proteolytic activity, on a TSA-milk plate containing 2 mM IPTG, of the mutant strain bearing pLAFR3, pAX24 (xcp gene cluster), pMMB190, or pMMB-P_{AQA} (P_{AQA}). (B) Immunoblot of cellular and extracellular protein extracts from the strains described for panel A and probed with LasB antiserum. C, cellular proteins; Ex, extracellular proteins.

In order to check whether the secretion observed with the couple XphA/XqhA is only dependent on these proteins or whether it requires additional secretion factors, the xphA and xqhA genes were overexpressed in a mutant strain deleted of the xcpRZ operon. In this genetic background, the P_{AQA} secretion subunit was unable to restore secretion, as shown by proteolytic assay on milk plates (Fig. 5A). However, complementation of the xcpRZ deletion by a plasmid bearing all the xcp genes caused the appearance of a clear hydrolysis halo as expected for a functional secretory process. This observation was confirmed by immunoblotting experiments showing that the XphA/XqhA pair is not able alone to promote secretion of LasB (Fig. 5B). Therefore, these results show that the P_{AQA} subunit requires the presence of XcpR-Z as additional secretion components to constitute a functional hybrid T2SS secretory apparatus.
strain bearing the empty vector pLAFR3 or the plasmid pAX24 containing all the xcp genes showed that expression of the xcpP and xcpQ genes restored the secretion of PaAP in the mutant strain (Fig. 7A and B). Therefore, it seems likely that although supporting the secretion of a majority of T2SS substrates, the PAQA subunit cannot promote the secretion of the aminopeptidase and that secretion of this protein is particularly XcpPQ specific. This interesting observation lends support to the idea that substrate recognition could imply different levels of specificity in direct relation not only with the XcpP/XcpQ pair but also with the XphA/XqhA pair.

Expression of xphA and xqhA genes. The xcpP and xcpQ genes of the classical T2SS of P. aeruginosa are known to be organized in an operon (1). It is likely that their homologues xphA and xqhA could also present the same genetic organization. Indeed, in silico analyses revealed an overlapping of the xphA and xqhA gene sequences. Moreover, a search for promoters using the BPROM software (www.softberry.com) indicated −10 and −35 boxes, respectively, 392 bp and 416 bp upstream of the initiation codon of xphA, and no additional such boxes could be found upstream of the xqhA gene. These observations strongly suggest the presence of only one putative promoter for the two genes and thus that, similarly to the xcpP and xcpQ genes, xphA and xqhA could be organized in an operon. However, nothing is known yet about the expression of these genes. Therefore, since xphA and xqhA genes are thought to be organized in an operon with a promoter localized upstream of xphA, an xphA-lacZ transcriptional fusion was con-

FIG. 6. Substrate specificity of the PAQA subunit. Extracellular proteins from PAO1 and the mutant strain ΔPOP₆O₆QA carrying the empty vector pMMB190 (p190) or pMMB-P₆O₆QA (P₆O₆QA) were analyzed by 2D-PAGE as described in Materials and Methods. Polarity of the IEF is indicated at the top of the gels, and the second-dimension migration is shown on the right (in kDa). Identified protein spots are indicated by arrows. Spot 1, PaAP; spot 2, AprA; spot 3, CbpD; spot 4, LasB; spot 5, PrpL.

FIG. 7. Secretion specificity of PaAP. Deletion of xcpP/xcpQ in the ΔPOP₆O₆QA mutant strain was complemented by introduction of pAX24 bearing all the xcp genes. Extracellular proteins were analyzed by 2D-PAGE under the same conditions as for Fig. 6. (A) Global protein profile of the secretome of the ΔPOP₆O₆QA strain carrying either pLAFR3 (empty vector) or pAX24. Squares highlight a small gel area containing PaAP. (B) Enlarged pictures of the squares shown in panel A. The arrow indicates the position of PaAP.
Control. Experiments. Gray squares, gene fusion; black diamonds, promoterless β-galactosidase activity of the xphA-lacZ fusion described in Materials and Methods. The strain expressing the transcriptional xphA-lacZ fusion was integrated in the chromosome of PAO1 as shown for β-galactosidase activity. (A) Growth curve. (B) β-galactosidase activity of the xphA-lacZ fusion. Results are expressed in Miller units and are means of duplicate samples from two independent experiments. Gray squares, gene fusion; black diamonds, promoterless control.

FIG. 8. Expression of the xphA-xqhA genes in PAO1. A transcriptional xphA-lacZ fusion was integrated in the chromosome of PAO1 as described in Materials and Methods. The strain expressing the transcriptional lacZ fusion and the control strain were grown in Luria-Bertani medium at 37°C, and samples were withdrawn at intervals and assayed for β-galactosidase activity. (A) Growth curve. (B) β-galactosidase activity of the xphA-lacZ fusion. Results are expressed in Miller units and are means of duplicate samples from two independent experiments. Gray squares, gene fusion; black diamonds, promoterless control.

Conclusions. *P. aeruginosa* is characterized by the existence of two independent T2SS, the Xcp and the Hxc systems, which are controlled, respectively, by quorum sensing (8) and by the phosphate concentration of the culture medium (2). The existence of the XqhA secretin and its involvement in the residual secretion observed in an xcpQ mutant was already reported by Martinez et al. (23). However, the presence of the XcpP homologue XphA and its role in the secretion process had not been described. Our results show that XphA is a newly described secretion protein that is normally produced by *P. aeruginosa*. Furthermore, the results also show that XphA works together with XqhA and further support the idea that these two proteins form an independent subunit within the T2SS that exerts various degrees of specificity towards a range of substrates. Regarding the species specificity of the T2SS, which has not been elucidated, the presence in the same bacterial species of proteins playing the same function although encoded by genes which are found at distant loci on the chromosome could contribute to additional insights on the determinants of the specificity of the T2SS.

Moreover, the significance of having two independent GspCD subunits in *P. aeruginosa* (XcpP/XcpQ and XphA/XqhA) remains unclear. Therefore, several hypotheses can be proposed concerning the physiological role of the P. AQA subunit in *P. aeruginosa*. A specific substrate, PaAP, has been found for XcpP/XcpQ, but no P. AQA-specific substrate has been discovered so far. It can thus be proposed that this secretory subunit is mainly involved in the secretion of substrates, which are produced under peculiar culture conditions (such as growth on solid medium or biofilms, for instance) or in response to an unknown signal occurring during host infection. Alternatively, since it is not quorum sensing regulated, the P. AQA subunit, in contrast to the XcpPQ one, can be involved at early growth stages by associating specifically with the Xcp proteins already produced in the exponential growth phase. Such an association leads to a hybrid secretory system which could secrete virulence factors at an early growth stage and help the establishment of *P. aeruginosa* in the host.

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