行政院國家科學委員會專題研究計畫　成果報告

十字花科黑腐病菌 Type II 傳送機器中 XpsG 蛋白與 XpsLMN 蛋白間交互關係之探討

計畫類別：個別型計畫
計畫編號：NSC93-2311-B-040-005-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學生化暨生物科技研究所

計畫主持人：陳凌雲

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 10 月 28 日

行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 □期中進度報告

（計畫名稱）

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 93－2311－B－040 －005－
執行期間：2004 年 08 月 01 日至 2005 年 07 月 31 日

計畫主持人：陳凌雲
共同主持人：
計畫參與人員：

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中　　華　　民　　國 94 年 10 月 28 日

中文摘要

XpsG蛋白是十字花科黑腐病菌第二型分泌機器中非常重要的組件，它與XpsH、I、J、K蛋白組裝成一個橫跨內外膜的類纖毛結構，以推動分泌性蛋白通過外膜。這種以XpsG蛋白為主體的類纖毛結構是否與第二型分泌機器的其他非類纖毛組件之間存在交互作用並不清楚，主要原因可能是來自於彼此交互作用是以動態或不穩定的型式進行。本研究中先以交連劑DSP處理完整細胞後，再以鎳離子親和性管柱與西方點墨法分析，結果發現XpsGh與XpsN (GspC)以及XpsD蛋白有交互作用。但當XpsN不存在時，則測不到XpsGh與XpsD的交互作用，然而如果增加XpsD蛋白的量，則XpsGh與XpsD的交互作用會有限的恢復。如果XpsD不存在時，則不會影響XpsGh/XpsN的交互作用。以免疫沉澱法證明XpsN與XpsD的交互作用與XpsG無關。上述結果對第二型分泌機器是以多蛋白協同進行蛋白分泌工作的想法提供有利的支持。

英文摘要

The major pseudopilin XpsG is an essential component of type II secretion apparatus of *Xanthomonas campestris* pv. campestris. Along with other ancillary pseudopilins, it forms a pilus-like structure spanning between cytoplasmic and outer membranes. Associations of pseudopilins with non-pseudopilin members of type II secretion apparatus were not well documented, probably due to their dynamic or unstable nature. In this study, by treating intact cells with a cleavable crosslinker dithiobis (succinimidylpropionate) (DSP), followed by metal chelating chromatography and immunoblotting on secretion-positive strains of *X. campestris* pv. campestris, we discovered associations of XpsGh with XpsN (GspC), as well as XpsD. However, chromosomal non-polar mutation in each gene exerted different effects upon the association between the other two. The XpsGh/XpsD association is undetectable in *xpsN* mutant; however, it was restored to a limited extent by overproducing XpsD protein. The XpsGh/XpsN association is unaltered by a lack of XpsD protein or an elevation of its abundance. Co-immune precipitation between XpsN and XpsD, while being independent of XpsG, was nonetheless enhanced by raising XpsG protein level. These observations agree with the proposition that the type II secretion apparatus in a cell may exist as an integrated multiprotein complex with all components working in concert. Moreover, in functional machinery, the association of the major pseudopilin XpsG with secretin XpsD appears strongly dependent on the existence of XpsN, the GspC protein.

前言、研究目的、文獻探討

Type II secretion (T2S) apparatus is utilized by a wide variety of pathogenic bacteria to secrete toxins or hydrolytic enzymes across outer membrane. Deficiency in any of the apparatus protein components may lead to an accumulation of secretory proteins in periplasm and a severe damage to the bacterial virulence. The processes involve a number of protein components in common, *i.e.* (i) secretin, known as D protein in T2S apparatus, that forms the secretion channel, (ii) the nucleotide-binding protein, known as E protein in T2S apparatus, (iii) a cytoplasmic membrane protein with three membrane-spanning sequences, known as F protein in T2S apparatus, and (iv) a group of prepilin-like proteins, also named pseudopilins for having an N-terminal signature sequence of Tfp precursor.

Pseudopilins have been proposed to form pilus-like structure spanning the cell envelope. G protein, being most abundant, is the major component of the pilus-like structure. The large sized form of *Xanthomonas campestris* pv. campestris XpsG protein detected in a secretion-positive

strain was proposed to derive, during cell breakage, from the pilus-like structure spanning between cytoplasmic and outer membranes. In addition to self-cross-linking, the major pseudopilin has been shown to cross-link with minor pseudopilins. Several possible roles have been proposed for the pilus-like structure in T2S. One is to act like a piston or a ratchet, by actively pushing or rotating, driving the secreted proteins to pass through the secretion channel. Repeated extension and retraction of Tfp required for twitching motility has been postulated for the pilus-like structure in T2S apparatus. A second role proposed for the pilus-like structure is to provide a scaffold, between cytoplasmic and outer membranes, probably guiding the secreted proteins to the secretion channel. The third possibility is for the central hollow space of the pilus-like structure serving as passage for secreted proteins.

Complementation of non-polar mutation in the out genes of Erwinia chrysanthimi with the out genes of a closely related species *Erwinia carotovora* revealed that all, except OutC and OutD, are replaceable. C and D proteins were thus postulated to be the gate-keepers of T2S apparatus. Direct association between *X. campestris* pv. campestris XpsN (GspC) and XpsD proteins were suggested by their co-immune precipitation. On the other hand, XpsN (GspC) protein was also shown to form a ternary complex with two other bitopic cytoplasmic membrane proteins XpsL and XpsM. The XpsL analogue in *V. cholerae*, EpsL protein, has been demonstrated to mediate the association of its nucleotide-binding protein, EpsE protein, with cytoplasmic membrane. Furthermore, the association of *E. chrysanthemi* OutF protein with OutL and OutE proteins was suggested from yeast two-hybrid studies and co-immune precipitation experiments. A quaternary complex comprising OutE, -F, -L, and -M was thus hypothesized to form a platform for the assembly of the pilus-like structure.

The first evidence for cross-talk between non-pseudopilin and pseudopilin components was from a genetic approach that implicates possible interaction between *P. aeruginosa* major pseudopilin $XcpT_G$ and its nucleotide-binding protein $XcpR_E$. Overproduction of *K. oxytoca* PulG caused a loss of PulC, -D, -E, -L, and -M. This has been taken as evidence for an interaction between PulG and these proteins. Furthermore, results from yeast two-hybrid system suggested an interaction between a minor pseudopilin OutJ and OutD, secretin of *E. chrysanthemi*, as well as OutL. However, no evidence has so far been available showing a direct connection between pseudopilin and non-pseudopilin protein components. In this study, by performing cross-linking, followed by metal chelating chromatography and immunoblotting analysis, we were able to detect, for the first time, an association of the major pseudopilin XpsG with the GspC protein XpsN and secretin XpsD in functional strains of *X. campestris* pv. campestris. In addition, analysis of pairwise interaction in mutant deficient in the third party indicated complex protein-protein interactions among the three components.

研究方法

Chemical Cross-linking

Preparation of Membrane Protein Extract

Metal Chelating Chromatography

Co-immune Precipitation

結果與討論

XpsG-containing Large Sized Molecules Generated from DSP Treatment of Intact Cells—Although cross-linked heterodimers of various combinations have been demonstrated

among four of the *P. aeruginosa* pseudopilins, no information is so far available regarding a direct association between pseudopilin and any non-pseudopilin components of T2S apparatus. To facilitate our search for possible interactive partners of the pilus-like structure, we examined three different disulfide-containing cross-linkers for their ability to cause dimerization of the major pseudopilin XpsG.

Preliminary results indicated that only DSP, but not DTSSP or DTBP, could cause XpsG to form large sized molecules that are sensitive to β-ME.

Presence of XpsN (GspC) and XpsD in XpsGh-containing Cross-linked Products—To determine whether any non-pseudopilin protein component, besides the major pseudopilin XpsG, might be present in the cross-linked products, we used a broad host-range vector-based clone pFGh that expresses a functional C-terminally His_6-tagged XpsG. To improve XpsGh binding to nickel, metal chelating chromatography was performed in the presence of 8 M urea. DSP cross-linking, followed by metal chelating chromatography of Triton X-100 extract of membrane proteins of XC1713(pFGh), revealed that only XpsN co-eluted with XpsGh; none of XpsL, XpsM, XpsE, or XpsF did. The association of XpsN with secretin XpsD, which constitutes the secretion channel in outer membrane, has been demonstrated with co-immune precipitation experiment. We thus are interested to find out if the XpsD is also present in the XpsG-containing cross-linked products. Immunoblotting with anti-XpsD antiserum revealed that XpsD co-eluted with XpsGh but not with untagged XpsG, suggesting that the two are likely to associate with each other in the cell.

Disappearance of Cross-linking between XpsGh and XpsD in xpsN Mutant Strain—To find out whether XpsN protein, known to interact with the major pseudopilin and secretin, is important in their association while other Xps proteins are present, we performed the same experiment examining the co-elution of XpsD with XpsGh in an *xpsN* mutant strain XC1709. The result clearly indicates that XpsD was no longer detectable in elute fractions when XpsGh was present. Co-elution of XpsD with XpsGh was apparently restored by introducing into XC1709 plasmid pHMNZ, which encodes the wild-type XpsN. It appears that XpsN is required for the association between XpsG and XpsD. However, by introducing extra copies of the *xpsD* gene encoded by plasmid pHM118 into *xpsN* mutant XC1709, we also detected XpsD co-eluting with XpsGh, albeit with much weaker signal than that detected in the complemented strain XC1713(pFGh). Previous experiments indicated that it was possible to detect the association between XpsGh and XpsD in a genetic background lacking all, except XpsO, T2S components. Here in the presence of all other components, XpsN turns out to be indispensable suggesting that certain Xps protein component(s) in the functional machinery may have made the association between XpsGh and XpsD an XpsN-dependent event. For instance, when XpsG exists in an assembly with all minor pseudopilins, its interactive relationship with XpsD could be altered so as to make XpsN a crucial factor.

Cross-linking between XpsGh and XpsN Neither Dependent on XpsD nor Enhanced by Raising Its Protein Level—The previous experiment indicates that the interaction between XpsGh and XpsD is strongly dependent on XpsN. Does the interaction between XpsGh and XpsN rely on XpsD? We performed the same type of cross-linking experiments, as described before, on an *xpsD* mutant strain XC1708 that was supplemented with plasmid pFGh encoding the His_6-tagged XpsGh. Only its total membrane extracts and concentrated elute fractions were compared with those in the complemented strain XC1713(pFGh). No apparent difference in the intensity of

XpsN signal was detected between the two eluted fractions. Moreover, introduction of plasmid pHM118 encoding the wild-type XpsD into *xpsD* mutant strain XC1708(pFGh) did not cause significant change in the intensity of XpsN signal present in the elute fractions, despite a 4.6-fold increase of XpsD protein in comparison with that produced from the chromosomal gene in XC1713(pFGh). These results suggest that neither the absence of secretin XpsD nor its overproduction resulted in any significant effect on the association between the major pseudopilin XpsG and XpsN (GspC).

Co-immune Precipitation between XpsN and XpsD Independent of, yet Improved by, More Abundant XpsG—Co-immune precipitation between XpsN and XpsD was detectable in the *xps* deletion strain XC17433. To examine the situation when all components, except XpsG, of T2S were present, we conducted co-immune precipitation, using anti-XpsN antiserum, on the *xpsG* mutant strain XC1713 in parallel with the wild-type strain XC1701. The co-precipitated XpsD protein signals detected with anti-XpsD antiserum appeared in nearly equal intensity, indicating their association being independent of XpsG. To further examine the effect of XpsG protein abundance on the association between XpsN and XpsD, we introduced extra copies of *xpsG* gene encoded on plasmid pFG into the wild-type strain XC1701, increasing the XpsG protein level by approximately 4-fold with normal secretion. Although it has been reported that overproduction of G protein caused secretion inhibition, a finite G protein abundance is permitted. *K. oxytoca* PulG protein, when raised 2–5-fold its normal abundance, did not cause any detectable inhibition in pullulanase secretion. Overproduction of XpsG at the level observed in the present study did not cause any obvious alteration in XpsD or XpsN protein abundance. However, the intensity of XpsD signal co-precipitated with XpsN was raised ~3.6-fold over that of the wild-type strain, indicating that the association between XpsD and XpsN can be enhanced by overproducing XpsG.