行政院國家科學委員會專題研究計畫 成果報告

奇異變形桿菌中 PhoP/PhoQ 傳遞系統調控表面移行，致病因子及 antimicrobial peptide 感受性之相關研究
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2320-B-002-070-
執 行 期 間 ：95 年 08 月 01 日至 96 年 12 月 31 日
執 行 單 位 ：國立臺灣大學醫學院醫學檢驗暨生物技術學系

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處 理 方 式 ：本計畫可公開查詢

中 華 民 國 97 年 02 月 22 日

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中英文摘要：

中文摘要

關鍵詞: 奇異變形桿菌、polymyxin B、細菌雙組成系統、lipopolysaccharide、移行能力、致病力

爲了了解奇異變形桿菌抗 polymyxin B(PB)的機轉，我們用 Tn5 mutagenesis 找到一突變株發現其 PB 感受性提高許多，序列分析發現 Tn5 插入一 細菌雙組成系統 response regulator 基因中，我們稱之 *rppA*，下游爲 histidine kinase *rppB*。接著我們建立一 *rppA* 剃除株，發現此剃除株之 lipopolysaccharide (LPS)會結合比野生株更多的 PB 且 SDS-PAGE profile 也不同於野生株。此外此剃除株展現較好的移行能力、細胞毒性，表現較多溶血素及鞭毛蛋白質。我們也觀察到 PB 可以經由 RppA 系統來影響移行能力、細胞毒性及溶血素和鞭毛蛋白質的表現。可知在奇異變形桿菌會透過 RppA 系統調控 PB 感受性、移行能力、細胞毒性及溶血素和鞭毛蛋白質的表現而 PB 是可能的訊號因子。這是第一次有關奇異變形桿菌中細菌雙組成系統調控 PB 感受性、移行能力及致病力的研究發現。

英文摘要

Keywords: RppA, *Proteus mirabilis*, polymyxin B, swarming, virulence

Proteus mirabilis, a human pathogen that frequently causes urinary tract infection, is intrinsically highly resistant to cationic antimicrobial peptides, such as polymyxin B (PB). To explore the mechanisms underlying *P. mirabilis* resistance to PB, a mutant which displayed increased sensitivity to PB (over160-fold) was identified by transposon mutagenesis. The mutant was found to have Tn*5* inserted in a novel gene *rppA*. Sequence analysis indicated that *rppA* may encode a response regulator of the two-component system and was located upstream of the *rppB* gene, which may encode a membrane sensor kinase. The *rppA*-knockout mutant of *P. mirabilis* had an altered lipopolysaccharide (LPS) profile. The LPS purified from the *rppA*-knockout mutant could bind more PB than that purified from the wild-type. These properties of the *rppA*-knockout mutant may contribute to its PB-sensitive phenotype. The *rppA*-knockout mutant exhibited higher swarming motility and cytotoxic activity, and expressed higher levels of flagellin and hemolysin than did the wild-type, suggesting that RppA negatively regulates swarming, hemolysin expression, and cytotoxic

activity in *P. mirabilis*. PB could modulate LPS synthesis/modification, swarming, hemolysin expression, and cytotoxic activity in *P. mirabilis* through an RppA-dependent pathway, suggesting that PB could serve as a signal to regulate RppA activity. Finally, we demonstrated that the expression of *rppA* was up-regulated by low-concentration of PB and down-regulated by high concentration of Mg^{2+}. Together, these data highlight the essential role of RppA in regulating PB susceptibility and virulence functions in *P. mirabilis*.

報告內容:

前言、研究目的、文獻探討

In a large number of bacterial species, the genes conferring resistance to Cationic antimicrobial polypeptides (CAP), including Polymyxin B (PB), are regulated by the bacterial two-component systems (11, 14, 15, 17-19). In *Salmonella enterica* serovar Typhimurium, evasion of CAP killing is regulated in part by the PmrA-PmrB two-component regulatory system (6, 7). PmrA-PmrB accomplishes resistance to CAP by up-regulating genes involved in covalent modifications of the LPS (6, 7). The LPS modifications reduce the negative charge of LPS and consequently decrease attraction and binding of CAP to the outer membrane. The PhoP-PhoQ two-component system, a master regulator of *S. enterica* serovar Typhimurium virulence functions, also has been shown to be involved in regulating resistance to CAP (4). PhoQ is an inner membrane sensor kinase composed of a periplasmic sensor domain and a cytoplasmic kinase domain that phosphorylates its cognate response regulator, PhoP, upon perception of specific environmental signals. The PhoP-PhoQ system is repressed by millimolar and activated by micromolar concentrations of magnesium (4, 5). The activation of PhoP-PhoQ increases the expression of PmrD (11), which in turn leads to the activation of PmrA (10), resulting in modification of LPS.

P. mirabilis is known to be highly resistant to the action of CAPs, such as PB (3, 9). The detailed mechanisms underlying *P. mirabilis* resistance to PB is not clear.

The swarming behavior of *P. mirabilis* is under the control of a complex regulatory network that may include bacterial two-component systems (12, 13, 23). It has been shown that LPS plays a critical role in swarming (16, 22) and that LPS modification affects both swarming and PB resistance in *P. mirabilis* (16). Moreover, activation of the PhoP-PhoQ two-component system, which is known to enhance CAP resistance, can lead to inhibition of swarming through repressing the expression of flagellin in *S. enterica* serovar Typhimurium (1). Together, these results suggest that swarming and CAP resistance may be co-regulated. In this study, we undertook a Tn*5* transposon mutagenesis approach to isolate super-swarming mutants of *P. mirabilis*. We identify a mutant with increased sensitivity to PB. The mutant was found to have Tn*5* inserted in

the *rppA* gene, a gene which may encode a response regulator of the two-component system.

研究方法、

Transposon mutagenesis and identification of the mutated gene. *P. mirabilis* swarming-aberrant mutants were isolated by mini-Tn*5* Cm (chloramphenicol) mutagenesis as described previously (12).

Gene-knockout by homologous recombination. Full length *rppA* including its promoter region was amplified by PCR using primers dA-1F and rppAR (Table 2) and cloned into pGEM®-T Easy (Promega) to generate pGrppA. The pGrppA was digested with *Xba*I and ligated with an *Xba*I-digested Ω (Km^r) gene cassette (20) to generate pGrppA-km, which contains the Km^r-inserted *rppA* gene.

Construction of the RppA-complemented strain. DNA fragment containing full length *rppA* and its promoter region was excised from pGrppA (see above) with *Sal*I and *Sph*I. The DNA fragment was ligated into a *Sal*I/*Sph*I-digested low-copy plasmid pACYC184 to generate the *rppA* complementation plasmid.

Real-time RT PCR. To study the effect of PB and Mg^{2+} on the expression of *rppA* mRNA, total RNA was extracted from cells using RNA-Bee™ kit. cDNA was obtained using Superscript II reverse transcriptase. The cDNA was then used as a template for real-time PCR.

MIC assay. In vitro determination of MIC for PB was performed according to the guidelines proposed by NCCLS.

Preparation and analysis of LPS. The LPS extraction and analysis were performed as described previously (21) with some modifications.

Swarming migration assay. The swarming migration assay was performed as described previously (8, 12).

Measurement of cell differentiation, flagellin level, and hemolysin activity. Preparation of cells for cell differentiation, hemolysin and flagellin assays was performed as described previously (12, 13).

Cytotoxicity assay. The experiments were performed as described previously (2) with some modifications.

結果

We identified a mutant, sw8, which was over 160 fold more sensitive to PB than the wild-type *P. mirabilis* N2. We found that Tn5 was inserted into a gene which we named *rppA*. The *rppA* gene and its downstream gene *rppB* was cloned and sequenced. *rppA* and *rppB* may encode a response regulator and a membrane sensor histidine

kinase, respectively, of the bacterial two-component signaling system.

The *P. mirabilis rppA*-knockout mutant has an altered LPS profile. The *rppA* mutant synthesized slightly more LPS than did the wild-type strain. The data indicate that the *rppA* mutant has undergone a qualitative change in LPS and this change causes the LPS from the *rppA* mutant to have higher binding activity to PB. PB can regulate the synthesis and modification of LPS in *P. mirabilis* and this regulation is mediated through an RppA-dependent pathway. The *rppA*-knockout mutant exhibited higher swarming motility and cytotoxic activity, and expressed higher levels of flagellin and hemolysin than did the wild-type, suggesting that RppA negatively regulates swarming, hemolysin expression, and cytotoxic activity in *P. mirabilis*. PB could modulate LPS synthesis/modification, swarming, hemolysin expression, and cytotoxic activity in *P. mirabilis* through an RppA-dependent pathway, suggesting that PB could serve as a signal to regulate RppA activity. Finally, we demonstrated that the expression of *rppA* was up-regulated by low-concentration of PB and down-regulated by high concentration of Mg^{2+}.

討論

These data highlight the essential role of RppA in regulating PB susceptibility and virulence functions in *P. mirabilis*. PB could serve as a signal to regulate RppA activity. Together these data suggest that low concentration of PB and possibly other CAPs can inhibit certain virulence functions of *P. mirabilis*. In this regards, it is tempting to suggest that CAPs secreted by epithelial cells of the urinary tract may still play roles in preventing *P. mirabilis* infection, even though the bacteria is known to be highly resistant to killing by PB and certain CAPs.

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計畫成果自評：

This is the first report that the bacterial two-component system regulates PB susceptibility and swarming in *P. mirabilis.* This study has been written and accepted by the Infection and Immunity Journal.