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題目：水禽敗血症巴氏桿菌 AROA 基因突變株之建立及抗原性分析[II]

（Construction and Antigenic Analysis of *aroA* mutants of *Pasteurella multocida* and *Riemerella anatipestifer* [II]）

一、中文摘要

本計劃中擬從本地分離株中挑選具高抗原性、高病原性之鴨瘟屢氏桿菌，利用核酸增幅、基因選殖、基因互補、及同源性基因交換等技術，來建構充分減毒但仍具足夠抗原性的 aroA 基因變異株。然而，經過數十次接合反應及十多次轉型作用並未獲得預期中的產物。

出血敗血性巴氏桿菌及鴨瘟屢氏桿菌感染症，也就是所謂的家禽霍亂及水禽傳染性漿膜炎，在水禽養殖業一直是一個嚴重的問題。現在已有疫苗來控制這兩個疾病，但是效果並不是非常好。大部分的菌苗含有一種或數種血清型的細菌，經福馬林不活化製成，通常只具部分保護效力；且菌苗曾經引起暫時性的內毒素性休克。目前以抑制數種自發性基因（*aro*A）來製成活毒減毒菌苗的方法已被採用。

關鍵詞：*aro*A **突變株、出血敗血性巴氏桿菌、鴨瘟屢氏桿菌**

Abstract

Pasteurella multocida (PM) and *Riemerella anatipestifer* (RA) infection, commonly known as fowl cholera and infectious serositis in waterfowl, continue to be a significant problem. Vaccines against both diseases are available but their efficacy has been poor. Most bacterins containing one or several serotypes of formalin-inactivated bacteria have induced protection. The bacterins also have caused transient endotoxin shock. The approach of attenuation based on several auxotrophic genes, such as *aro*A, has been employed to make live attenuated bacterins. The *aroA* gene of PM & RA will be cloned by complementation of the *E. coli* mutant after electroporation with a genomic library constructed in plasmid. Their sequences will be determined by Sanger's dideoxy method. The genes will be inactivated by insertion of a antibiotic resistance gene and reintroduced by allelic exchange into the chromosome of the parental PM & RA. The mutants' virulence will be examined in a mouse model.

Keywords: *aro*A mutant, *Pasteurella multocida*, *Riemerella anatipestifer*

二、緣由與目的

由出血敗血性巴氏桿菌（*Pasteurella multocida*）所引起的家禽霍亂（fowl cholera**）為家禽，尤其是鴨、鵝等水禽類之重要傳染病**[1, 8]**，此病的特徵為：急性型呈敗血症及嚴重下痢：慢性型發生水腫及關節炎。另外由鴨瘟縷氏桿菌（*Riemerella anatipestifer*）引起鴨、鵝敗血症及傳染性漿膜炎，在水禽類亦普遍發生，造成嚴重的危害**[2, 16, 21, 22, 25, 26]。**針對這二種病雖然已有許多活菌或死菌菌苗被開發出來，但這些菌苗仍未能完全有效的控制家禽霍亂之為害**[14]。

一般以溫度變異株（temperature-sensitive mutant; TS mutant**）來製造活菌菌苗**[7, 12, 27]。**除此之外，利用某些營養缺陷型變異株（**auxotroph**）可維持恆定，不**

發生變異回復（reversion），且發生減毒的特性，很多研究者均朝此減毒或無毒活菌菌苗的方向發展[4,15]。這些基因包括 *aroA*, *crp*, *cya*, *gal*E, *hem*A, *htr*A, *omp*R, *pho*P, *ser*C 等，獲得具體保護效力的例子如：鼠傷寒沙門氏桿菌（*Salmonella typhimurium*）[9, 17]、傷寒沙門氏桿菌（*Salmonella typhi*）[12, 28]、豬霍亂沙門氏桿菌（*Salmonella cholerasuis*）[19]、赤痢志賀菌（*Shigella flexerneri*）[30]、百日咳博氏桿菌（*Bordetella pertussis*）[24]、腸炎耶氏桿菌（*Yersinia enterocolitica*）[3, 20]、炭疽桿菌（*Bacillus antrracis*）[13]、出血敗血性巴氏桿菌（*Pasteurella multocida*）[10]、淋病球菌（*Neiseria gonorrhoeaea*）[5]、溶血巴氏桿菌（*Pasteurella haemolytica*）[11, 18, 29]等。其中沙門氏桿菌也被用來當作載體，接入大腸菌腸毒素、霍亂弧菌 O 抗原、破傷風毒素等其他抗原基因，而形成多價菌苗[17]。*aro*A 基因的產物為 3-enolpyruvaylshikimate-5-phosphate synthetase 酵素，缺此酵素則無法合成分支酸（chorismate），繼而導致其一：無法製造 phenylalanine, tryptophan, tyrosine 等芳香族氨基酸，然而這些氨基酸在動物體內可供應，因此變異株的生長不受影響。繼而導致其二：無法製造 PABA, 2,3 dihydrobenzoic acid 等重要中間物，這些中間物可繼續合成重要之葉酸（folate）及 enterochelin；而動物體內並不存在這些產物。另外基因變異也會導致其三：無法製造維生素 K 及 Q 輔脢（ubiquinone），但是這二種先驅物對細菌之存活並不重要。利用上述特性，即由於 *aro*A 變異株在動物體內發育差，但是可存活，並且很可能仍然會表現某些緊迫蛋白（stress protein）例如熱休克蛋白（heat shock protein）、攜鐵蛋白（siderophore）；某些外膜蛋白（outer membrane protein）、粘著素（adhesin）、integrin；或少量毒素等毒力因子[8]；能引發更強固之細胞性免疫，因此很適合作為活菌苗菌株。

本計畫擬針對出血敗血性巴氏桿菌及鴨瘟縷氏桿菌進行建構 *aro*A 變異株。首先收集野外分離，確定高病原性之菌種。以 complementation 的方式選殖 *aro*A 基因；植入抗生素抗性基因；再以電氣轉形之方法植入出血敗血性巴氏桿菌及鴨瘟縷氏桿菌。如此便可獲得 *aroA* 基因變異株。初步將進行小白鼠減毒試驗；第二年則評估製成活菌菌苗之可行性。

三、結果與討論

基因庫選殖依下列步驟並未獲得任何陽性反應 1. 於 eppendorf 中加入 1 μL T4 DNA ligase、1 μL 10X ligation buffer 與切妥之 vector 2 μL (50 ng)，隨後加入 PM 或 RA 經限制 切割產物 (調整使 vector:insert 之濃度/分子量比為 1:3) 及無菌水使反應體積為 10 μL。2. 置於 14 ℃ 水浴隔夜，真空抽乾及酒精洗後以 5 μL 水溶解，稍加離心後置冰上。3. 取 40 μL AB2829 cell，加入 1 μL 之 ligation mixture 並攪拌混合。4. 依前述條件進行電氣轉形。加入 960 μL Minimal medium 培養基，隨後置於 37℃ 下以轉速 225 rpm 振盪 1 小時後取出放冰上。5. 各取 50 μL 與 100 μL 塗抹在含 100 μg/mL ampicilin agar plate 上，37℃ 培養至少 18 小時。6. 長出之菌落即含 PM 或 RA 之 *aro*A 基因。

四、計畫成果自評

執行本計畫第一年後顯示，以互補方式進行 a*aro*A 基因選殖，再進一步建構水禽巴氏桿菌（PM 或 RA）a*aro*A 基因突變株的構想雖可行。但顯然不太容易。惟在一年的實驗中讓研究生學會了一般分子生物學的技術也算是一大收穫。第二年雖已改變為直接增幅法，很遺憾仍未能有所突破。

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