行政院國家科學委員會專題研究計畫 期中進度報告

以標籤式突變法分析 Salmonella choleraesuis 活體內毒力因子(1/3)

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計畫主持人：張照夫

計畫參與人員：張照夫，顧有為

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計畫名稱：以標籤式突變法分析 *Salmonella choleraesuis* 活體內毒力因子(I):

***Salmonella choleraesuis* 突變庫製備(1/3)**

計畫編號：NSC 91-2313-B-002-386

執行期限：91 年 8 月 1 日至 92 年 7 月 31 日

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主持人：張照夫

電子信箱：cfchang@ntu.edu.tw

執行機構及單位: 國立台灣大學獸醫學系

中文摘要

豬霍亂沙門氏菌 (*Salmonella choleraesuis)* 是兼性細胞內寄生性病原，能引起豬隻敗血症及腸炎，死亡率甚高，為養豬業重要之傳染病，經濟損失嚴重。本菌偶亦引發人類之敗血症、腸炎及骨髓炎，為人畜共通傳染病原，威脅公共衛生安全。

過去對細菌毒力因子之分子生物學研究，都以試管內 (*in vitro*) 毒力基因表現為研究方法。試管內表現之毒力因子與病原菌在動物宿主活體內 (*in vitro*) 表現的基因產物，未必相同。因此以試管內基因表現技術研究細菌之致病機制，仍有許多盲點無法克服。本計畫擬以標籤式突變法分析 *S. choleraesuis* 在豬活體內表現之毒力因子，分三年進行。第一年為突變庫製備。以墨點雜合法篩檢 282 個被 Bio-dot 濾過裝置固定在 Hybond N^+尼龍膜之質體。為檢查是否為交叉雜合產生的雜合訊號，以 94 個質體之標籤作成的探針雜合 282 質體，將有交叉雜合的質體刪除。 其餘的質體以相同的方式持續進行雜合，作為探針質體之數目則遞減為 48、24 和 8。 結果得到 82 個 pUT mini-Tn5Km2 質體含有獨特標籤序列。一組 48 個含有強烈雜合訊號標籤之質體被重複應用構築成 20 個 *S. choleraesuis* 突變庫。

關鍵詞：豬霍亂沙門氏菌，標籤式突變法，活體內毒力因子

Genetic analysis of the *in vivo* virulence factors by signature-tagged mutagenesis

(I): Generation of the *Salmonella choleraesuis* mutant bank(1/3)

Chao-Fu Chang

Department of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

ABSTRACT

Salmonella choleraesuis is a facultative intracellular pathogen that causes a septicemic disease of pigs. *S. choleraesuis* is host-adapted for swine. *S. choleraesuis* occasionally also causes human infection, so it is a zoonotic pathogen. Although *S. choleraesuis* causes an economic loss in swine industry, the information about the virulence genes of this organism is very limited. The hypothesis is that there are many proteins which are only present when the bacteria are growing in the animal host (*in vivo*) and not when the pathogen is grown under laboratory conditions (*in vitro*), and that these proteins play a critical role in the disease process. The goal is to identify genes encoding potential virulence factors that are expressed only *in vivo* in the swine host. These proteins may prove to be important in the pathogenesis of *S. choleraesuis* infection in pigs. Generation of the *S. choleraesuis* mutant bank was the purpose of the first year project. A series of identical membranes for dot blot hybridizations was prepared by transferring of 282 plasmids onto Hybond N^+ membranes using the Bio-dot Microfiltration Apparatus. To test whether the hybridization signals resulted from cross-hybridization between tags, amplified tags from 94 plasmids were used to probe a dot blot of 282 plasmids, which included the 94 used to generate the probe. There were cross-hybridization to the probe. These plasmids were deleted. The same method was repeatedly for the other plasmid. Cross-hybridization was continued but the amount of plasmid was used to probe were 48, 24 and 8. Eighty-two pUT mini-Tn5Km2 plasmids containing unique tag were found. A set of 48 plasmids containing strongly-hybridizing tags was repeatedly used to construct 20 libraries of *S. choleraesuis*.

Keywords: *Salmonella choleraesuis*, signature-tagged mutagenesis, in vivo virulence factor

INTRODUCTION

Salmonella choleraesuis is a facultative intracellular pathogen that causes a septicemic disease of pigs. *S. choleraesuis* is host-adapted for swine. *S. choleraesuis* occasionally also causes human infection, so it is a zoonotic pathogen. Although *S. choleraesuis* causes an economic loss in swine industry, the information about the virulence genes of this organism is very limited. The hypothesis is that there are many proteins which are only present when the bacteria are growing in the animal host (*in vivo*) and not when the pathogen is grown under laboratory conditions (*in vitro*), and that these proteins play a critical role in the disease process. The goal is to identify genes encoding potential virulence factors that are expressed only *in vivo* in the swine host. These proteins may prove to be important in the pathogenesis of *S. choleraesuis* infection in pigs.

Generation of the *S. choleraesuis* mutant bank was the work of the first year project. Transposons are genetic elements that can jump to new locations. They typically have terminal inverted repeats, encode a transponase, and move by excising themselves from a donor site and reinserting elsewhere. Transposon insertions can alter the regulation and expression of genes. The identification of virulence genes and infection mechanisms in bacterial pathogens can lead to new insights for disease-control strategies. Transposons can be used to create disruptions in important genes for pathogenicity.

Signature-tagged mutagenesis(STM) uses transposons containing unique PCR-amplifiable oligonucleotide regions that serve as tags for each transposon. The transposons are mobilized within a bacteria pathogen and one thousand transposon-generated mutants are arrayed and then pooled. The pool is used to inoculate the host organism. Following recovery of bacteria from the infected organism, the oligonucleiotide regions are amplified from the pooled transposon tags and used to probe the original array. Lack of a particular tag in the recovery pool correlates to lack of survival of a certain clone within the host. The method relies on mutants being able to survive and replicate in vivo as mixed populations and identifies genes necessary for growth in the host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are described in Table 1.

Bacteria were grown on Luria-Bertani (LB) medium unless otherwise specified with additional antibiotics where appropriate at the following concentrations: nalidixic acid (nal), 25 mg mL^{-1}; kanamycin (kan), 100 mg mL^{-1}. Minimal media consisted of M9 salts supplemented with 1% glucose and 1 mM nicotinamide.

Selection of signature tags

Signature tags were amplified and labeled as described previously (Hensel et al., 1995) with the following modifications. Tags were initially amplified from plasmid DNA (5 μg) in the presence of nonradioactive dNTPs (200 mmol each). Precipitate PCR products with ethanol. Resuspend DNA in 20 μL TE and purify by electrophoresis through a 1% gel in TAE buffer. Excise gel slices containing fragments of about 80 bp and extract by Gel extraction kit (Qiagen). All reactions were performed in a Perkin Elmer 9700 Thermal cycler. DNA dot blot hybridization analysis to the plasmids carrying the 48 different tags on filter.

Mating.

A wild nalidixic acid-resistant strain CN214 was obtained by plating to solid media containing nal. Matings were performed by mixing equal volumes (0.5 mL) of overnight liquid cultures of the donor (S17-1 pir harbouring pUTmini-Tn5Km2 derivatives;40000 cfu) and the recipient (CN214nal^r;OD_{580} of 1.0) on M9 salts agar without antibiotics. The bacteria were incubated for 16 h at 30℃, harvested from the plates with a sterile 10 ml LB, and streaked to selective media
containing kan and nal. Exconjugates were identified after 18 h growth at 37℃, and tested for resistance to chloramphenicol by replica plating before use in further experiments.

RESULTS

Construction of library of tagged

E. coli CC118- λ pir was transformed with pUTmini-Tn5Km2 containing signature tags (de Lorenzo et al.,1990; Hensel et al., 1995). A total of 282 transformants (Fig. 1) was screened to identify 82 plasmids harbouring tags that gave consistent, specific signals. Dot blots of the transformants were probed using all the tags labeled with [α -^{32}P]-dCTP. The remainder were analyzed for cross-hybridization (Fig. 2) by probing the blots with tags amplified from subsets of the transformants.

Construction and characterization of the mutant library

Forty-eight uniquely tagged pUTmini-Tn5Km2 derivatives were used to construct the mutant library. The plasmids were transformed into the donor strain, S17-1 lpir, and then used in independent matings with the *Salmonella choleraesuis* recipient, CN214. Pick individual chloramphenicol resistance exconjugation into 96-well microtiter dishs containing LB medium. Grow overnight with gentle shaking at 37℃. 50 μL of 75% glycerol was added to each well, and plates were stored frozen at -80℃. A total of 960 mutants were arrayed into 20 pools each containing 48 mutants.

DISCUSSION

A total of 282 tags were screened by dot blot hybridization with their corresponding [^{32}P]-dCTP-labeled to identify 279 tags that amplified and labeled efficiently. These tags were then tested for cross-hybridization and 82 pUT mini-Tn5Km2 plasmids were containing unique tag. DNA sequence of tags did not share more than 50% identity over the variable region was sufficient to prevent cross-hybridzation (Hensel et al., 1995). cross-hybridzation was necessary for selection of tags.

There are two factors that restrict the complexity of pools of different mutants for use as inocula in infection studies. First, the pools were too complex and that strains with unimpaired colonization ability (Chiang and Mekalanos 1998). Second, in hybridization analysis, the pools were too complex and hybridization signals will become too weak to be detected (Hensel et al., 1995).

STM was divided into steps: the construction of library of tagged mutants and the in

vivo screening of the library. We think that we will get good results.

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Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Source
Escherichia coli		
DH5 α	*F` endA1 supE44 thi-1 hsdR17(r_K^- m_K^+)recA1 gyrA relA1 Δ(lacIZYA-argF)U169deoR (ϕ80 dLac Δ(lacZ)M15)*	Gibco-BRL
S17-1 λpir	*recA thi pro hsdR2M1 RP4::2-Tc::Mu::Km Tn7 lysogenized with λpir phage*	Miller and Mekalanos (1988)
CC118 λ*pir*	*Δ(are-leu) araD DlacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1 lysogenized with lpir phage*	de Lorenzo et al. (1990)
Salmonella choleraesuis		
CN214	*Km^s Cam^r Nal^r*	This Lab.
Plasmids		
pUTmini-Tn5Km2		Hensel et al. (1995)

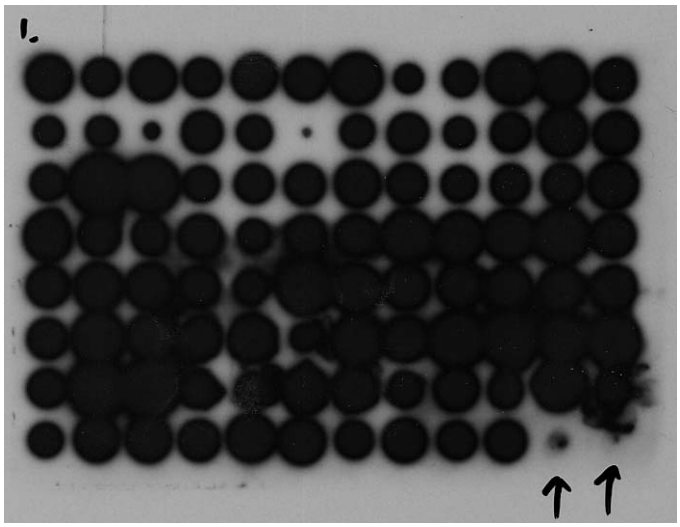

Fig. 1. *Eseherichia coli* strains CC118 *λpir* was transformed with pUTmini-Tn5Km2 containing signature tags. Transformants were screened by dot blot to identify plasmids harbouring tags that gave signals. Arrows are negative control.

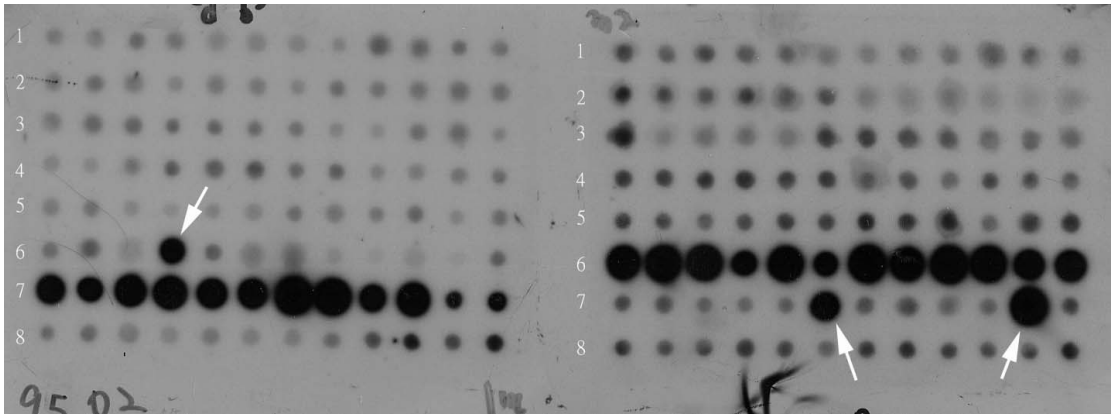

Fig. 2. Detection of cross-hybridization by dot blot hybridization analysis. Dot blots were probed using 12 tags (seventh and sixth line) labeled with [^{32}p]-dCTP. Plasmids that were cross-hybridization (white arrow) were discarded.