

行政院國家科學委員會專題研究計畫成果報告

草綠色鏈球菌之 *groEL* 基因定序與臨床應用(2/2)

Sequencing of *groEL* genes of viridans group streptococci and application on species identification

計畫編號：NSC 90-2320-B-002-188

執行期限：90 年 8 月 1 日至 91 年 7 月 31 日

主持人：鄧麗珍 國立台灣大學醫學院 醫事技術學系

計畫參與人員：陳品妘 國立台灣大學醫學院 醫事技術學系

一、中英文摘要

草綠色鏈球菌 (viridans group streptococci) 為人體口腔內之正常菌叢，但卻也是齲齒、感染性心內膜炎、敗血症及許多深部組織嚴重感染之重要病原菌。草綠色鏈球菌是一群相當異質 (heterogeneous) 的細菌，依據 16S rRNA 基因序列將草綠色鏈球菌分為 5 群：the milleri (anginosus) group, the mitis group, the mutans group, the salivarius and the bovis group。由於不同菌種之感染部位、致病性、抗藥性均有差異，但在臨床鑑定上並沒有一套很簡易且準確的方法，常造成困擾。因此利用分生方法發展快速鑑定方法一直是學者的目標。本計劃已完成四株 ATCC 菌種 *groESL* 基因全長定序，及六株 ATCC 菌種 *groESL* 基因近乎全長定序。且分析各草綠色鏈球菌之間的 *groESL* 基因親屬遠近關係，並發現 *groES* 與 *groEL* 之間的 spacer 序列可作為草綠色鏈球菌的快速區分方法 (已發表於 J Clin Microbiol 40:3172-3178) (如附件)。

關鍵詞：草綠色鏈球菌、*groESL* 基因、序列分析、快速鑑定

Abstract

Viridans group streptococci (VGS) currently encompass 20 species, which are members of the normal mouth flora and are a major cause of endocarditis or other diseases.

Classification depends on a combination of features including pattern of hemolysis observed on blood agar plates, antigenic composition, growth characteristics, biochemical reactions, and more recently, genetic analysis. On the basis of 16S rRNA sequence homology, these bacteria are categorized in five groups: the milleri (anginosus) group, the mitis group, the mutans group, the salivarius and the bovis group. No single system of classification suffices for the identification of these heterogeneous organisms. The *groESL* genes (also known as *cpn10/60*), is ubiquitous and highly conserved among bacteria. In this study, the full-length sequences of *groESL* genes (*cpn10/60*) from *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus gordonii*, and *Streptococcus sanguis*, and the near full-length sequence of the *groESL* genes of *Streptococcus intermedius*, *Streptococcus bovis*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, and *Streptococcus salivarius* were determined. Phylogenetic analysis of *groES* and *groEL* genes yielded evolutionary trees similar to the tree constructed with the 16S rDNA. Intraspecies variation of the spacer for some species was minimal on clinical isolates. The *groESL* sequence data provide an additional parameter for viridans streptococcal species identification. (published on: J Clin Microbiol 40:3172-3178)

Keywords: viridans group streptococci, *groESL* genes, sequencing

二、緣由與目的

草綠色鏈球菌為人體口腔中主要的正常菌群 (normal flora)，但也是亞急性細菌性心內膜炎、菌血症、及許多深部組織嚴重感染之重要病原菌^(8,9)。草綠色鏈球菌是一群相當異質 (heterogeneous) 的細菌，至少有 20 種以上的菌種 (species)，沒有單一系統可以來分類草綠色鏈球菌。一般依照菌落生長特徵、在血液培養基的溶血型式、細胞壁抗原組成、生化反應^(13, 18, 53)和最近發展的分子生物學方法，如 DNA-DNA hybridization、DNA-rRNA hybridization 和 small subunit (16S) rRNA 基因序列等來分類。依據核糖體核糖核酸基因 (16S rRNA 基因) 序列可將草綠色鏈球菌分成 4 群^(6, 30)，此分類為大部分學者所接受。包括 the anginosus group，其中包含 *S. anginosus*、*S. constellatus* 及 *S. intermedius*；the mitis group，其中包含 *S. sanguis*、*S. parasanguis*、*S. gordonii*、*S. oralis*、*S. mitis* 及 *S. pneumoniae*；the mutans group，菌種主要有 *S. mutans*、*S. criceti*、*S. rattii* 及 *S. sobrinus*；the salivarius group，其中包含 *S. salivarius*、*S. thermophilus* 及 *S. vestibularis*。在 mitis group 中常見的致病菌——肺炎鏈球菌 (*Streptococcus pneumoniae*)，雖然其生長特徵顯然不同於其它草綠色鏈球菌，但在 16S rRNA 基因層面上 (genetic level) 和 *S. oralis*、*S. mitis* 非常相似。因此，*S. pneumoniae* 並不歸於草綠色鏈球菌。此外，也有人把 *S. mitis*

group 細分成兩群——*S. sanguis* group 和 *S. mitis* group。草綠色鏈球菌不同菌種間的生化特性、抗藥性及引起的疾病並不太相同，例如，*S. mitis* group 中的 *S. sanguis*、*S. gordonii* 和 *S. oralis* 易引起感染性心內膜炎⁽¹⁴⁾，也易在牙齒形成牙斑 (dental plaque)。*S. mutans* 最易引起齲齒。The anginosus group (或稱為 *milleri* group) 易引起膿瘍，其中 *S. intermedius* 易引起腦、肝膿瘍，*S. anginosus* 易在泌尿生殖道發現^(19, 52)。此外，近 10 年來，草綠色鏈球菌對青黴素 (penicillin) 的抗藥性也逐漸增加中^(11, 47)，尤其以 *S. mitis* 和 *S. oralis* 最多。因此，無論是探討致病因子、流行病學或臨床的治療方面，正確的菌種鑑定是有意義且必要的，尤其在感染性心內膜炎的復發以及評估血液培養陽性 (positive blood cultures) 對病人的影響均很重要⁽¹¹⁾。近年來草綠色鏈球菌雖然已有較清楚的分類，但是在臨床實驗室的檢驗方法仍然相當混亂，經常會碰到很難鑑定的菌株。臨床上草綠色鏈球菌臨床的菌種鑑定方法，仍以傳統試驗為標準^(5, 53)，但所需時間極長，並不實際。因此許多實驗室利用快速鑑定⁽⁴⁾或自動化鑑定系統，例如 API 20S、API Rapid ID 32 Strep (bioMérieux-Vitek Inc.)、Vitek GPI card、MicroScan GP 等。API Rapid ID 32 Strep 由 32 種試驗 (tests) 組成，接種濃度 McFarland No.4 的菌液後，4 小時判讀，此系統的資料庫 (database) 較大，也包含一些新近命名的草綠色鏈球菌，如 *S. gordonii* 和 *S. oralis*⁽³²⁾，操作簡單快速；但是用在草綠色鏈球菌上，卻經常有下列問題：第一，

有些細菌會因生化特性的改變 (phenotype shifts) 導致重複試驗有不同結果, 而無法鑑定出正確的菌種⁽⁴⁶⁾。第二, 相同的草綠色鏈球菌菌種, 可能會有不同的生化特性表現, 造成鑑定上的困難^(5, 32)。第三, 命名系統的變更, 例如 *S. milleri* group 中的 3 個菌種, 在 1991 年才有正式的命名及特徵描述為大家所接受^(50, 54); *S. mitis* group 中的 *S. sanguis*、*S. mitis* 及 *S. oralis* 在 1989 年有更新的特徵描述⁽³²⁾; 加上歐美系統在 *S. milleri* group 及 *S. mitis* group 命名意見的不同, 近年來又有許多菌種陸續發現及命名, 細菌特徵的更新不能適時出現在資料庫, 更造成鑑定上的複雜性。根據大規模的研究調查, API Rapid ID 32 Strep 是鑑定正確率較高的商業化產品, 正確率約 80%, 其餘 20% 為鑑定錯誤或鑑定不出來的菌株, 其中以 *S. mitis* group 和 *S. milleri* group 錯誤率較高^(3, 20, 27, 31)。有鑑於此, 近年來, 有許多替代方法被發表, 例如, 菌體脂肪酸 (cellular fatty acids) 分析⁽¹²⁾, 蛋白質電泳 (SDS-PAGE) 分型、PCR 或去氧核糖核酸雜交^(2, 7, 17, 21, 26, 31, 38, 41, 42)、或利用 16S rRNA 基因來鑑定^(26, 29, 33, 39, 40, 41, 51)。熱刺激蛋白 (Heat shock proteins, HSPs) 普遍存在於細菌以至於人類, 當細菌遭遇環境壓力下, 如熱刺激、養份不足、毒素等, 熱刺激蛋白會大量表現, 統稱為壓力蛋白 (stress proteins)⁽¹⁶⁾。傳統上, 依據分子量的不同, 壓力蛋白分為 5 群⁽¹⁶⁾ (five families) —HSP90 (包含 HtpG)、HSP70 (包含 DnaK)、HSP60 (包含 GroEL 和 GroES)、small HSPs (HSPs 15-30) 和 other

HSPs (包含 DnaJ 和 GrpE); 其中, HSP60 又被稱為 chaperonins (cpns, cpn60 和 cpn10), 它和蛋白質的穩定性有關。*groEL* 基因也被發現與核糖體核糖核酸基因有著類似的特性, 在各生物間, 例如細菌、真菌、以至於哺乳動物, 都有部分高度相似性 (highly conserved) 及部分變異性 (variable 的區域^(15, 49)), 相似性程度比 16S rRNA 基因稍低, 也可當作分類或鑑定的工具^(34, 35, 36)。在 1989 年, 有學者以片段的 65 kDa 分枝桿菌抗原基因 (65 kDa mycobacterial antigen gene, *groEL*) 當作探針 (probe) 來鑑定生長緩慢的分枝桿菌 (*Mycobacterium* species)^(25, 37)。1996 年, Goh 等人以片段 *groEL* 基因當作探針, 進行點漬雜交法 (dot blot hybridization), 用來鑑定葡萄球菌 (*Staphylococcus* species)^(23, 24), 1998 年應用於鑑定 *Streptococcus iniae*⁽²²⁾, 結果具有相當高的準確性。因此, 本計劃也想藉由 *groEL* 基因層面來分析各草綠色鏈球菌之間的親屬遠近關係, 並發展出草綠色鏈球菌的快速鑑定方法。根據已發表細菌的 *groEL* 基因相似性的序列, 設計出一對引子 (primers) —GroEL-F 和 GroEL-R, 對草綠色鏈球菌進行 PCR 反應, 得到 *S. gordonii* 之片段 *groEL* 基因, 約 575 base pairs (bp)⁽¹⁾。再根據此片段基因序列設計出另一對引子 —Gor-2F 和 Gor-2R 進行 PCR 反應, 而製備出其它大部分草綠色鏈球菌之片段 *groEL* 基因進行比對⁽¹⁾。本計劃藉由已知的片段 *groEL* 基因序列去選殖整段 *groESL* 基因, 分析基因序列構造, 探討菌種間演化關係, 並應用於

臨床上快速鑑定。

三、結果

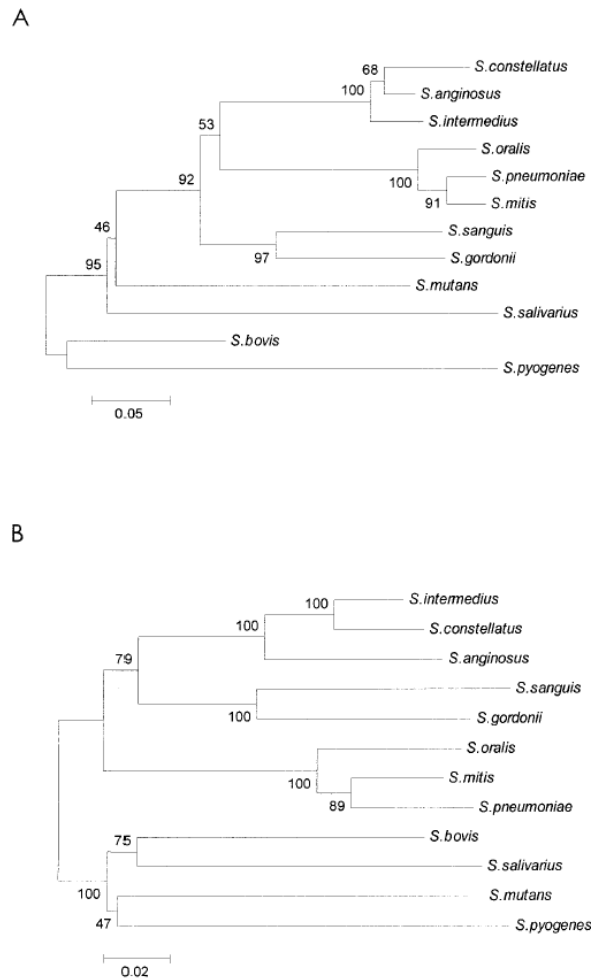
LA-PCR. 利用 LA PCR *in vitro* cloning 的方法，接上適當的 linker，進行草綠色鏈球菌四株 ATCC 參考菌株：*S. anginosus*、*S. constellatus*、*S. gordonii* 與 *S. sanguis* 之 *groESL* 全長基因選殖及定序。得到 *groESL* 基因全長序列。

Sequencing. 已完成四株 ATCC 草綠色鏈球菌 *S. anginosus*、*S. constellatus*、*S. gordonii* 與 *S. sanguis* 的完整(full-length) *groESL* 序列，及六株 ATCC 草綠色鏈球菌 *groESL* 基因近乎全長序列。完整(full-length) *groESL* 序列均包括 *groES* (282-288 bp)、spacer (15 至 111 bp)、與 *groEL* (1,623 bp)。鏈球菌的 *groESL* 基因序列經由比對後，發現與其他革蘭氏陽性細菌類似，在啟動子區域有調控基因序列-CIRCE。在 *groES* 與 *groEL* 之間有一段 spacer 的區域，各個菌種之間的長度不盡相同。而在 *S. gordonii* *groESL* 基因後面有長的反重複序列 (inverted repeat, IR)，可能為基因轉錄停止訊號 (rho-independent transcriptional terminator)。

Nucleotide sequence accession numbers. The nucleotide sequences of *groESL* genes determined in this study were deposited in the GenBank sequence database. The accession numbers for the full-length *groESL* genes are: *S. anginosus*, AF378195; *S. constellatus*, AF378196; *S. gordonii*, AF338228; and *S. sanguis*, AF378197. And the accession numbers for the nearly full-length *groESL* genes are: *S. bovis*, AF389514; *S. intermedius*, AF389515; *S. mitis*, AF417589; *S. mutans*, AF389516; *S. oralis*, AY38047; and *S. salivarius*, AF389517.

Phylogenetic relationships. 分別分析鏈球菌的 *groES*、*groEL* 基因的分化樹狀圖。結果如圖一。

FIG. 1. Phylogenetic relationships of various species of *VCS*, *S. proteomacrus*, and *S. pyogenes* based on the nucleotide sequences of the *groES* and *groEL* genes. The phylogenetic tree was generated by the neighbor-joining method within the MEGA package. The numbers at the nodes are the percentages of occurrence in 500 bootstrap resamplings. (A) Phylogenetic tree showing relationships of *groES* genes; (B) phylogenetic tree showing relationships of *groEL* genes.



圖一、草綠色鏈球菌 *groESL* 基因演化關係。(A) GroES (B) GroEL

四、討論

本計劃已完成四株 ATCC 草綠色鏈球菌 *S. anginosus*、*S. constellatus*、*S. gordonii* 與 *S. sanguis* 的完整(full-length) *groESL* 序列，及六株 ATCC 草綠色鏈球菌 *groESL* 基因近乎全長序列完成。經比對後，草綠色鏈球菌 *groESL* 基因類似其它革蘭氏陽性細菌的 *groESL* 基因或 *dnaK* 基因，啟動子區域有類似 σ^{32} 的 DNA 序列，並有 inverted repeat (IR) 結構，其為 TTAGCACTC-N₉-GAGTGCTAA。此結構稱為 CIRCE (controlling inverted repeat of chaperone expression)，在非熱刺激的情形

下,有 repressor 結合上 IR,可以抑制 *dnaK* 或 *groESL* 的表現。

在 GroEL 蛋白質尾端,革蘭氏陰性細菌普遍存在許多 Methionine (M) 和 Glycine (G) 這 2 種胺基酸,且存在 GGM 3 個胺基酸的重覆現象 (GGM repeat)。但革蘭氏陽性細菌比較不一樣,*S. epidermidis* GroEL 蛋白質尾端胺基酸序列為 PGMGGMPGMM, *S. pyogenes* 為 PGMMGGF, 而 *S. gordonii* 為 PSMMGGMM。

GroES 與 GroEL 間有一小段序列,稱為 spacer。Spacer 的長度與序列似乎與菌種有關。我們已測試一些臨床菌株,大致上均與參考菌株的結果符合。只有 *S. mitis* 與 *S. oralis* 仍無法以 spacer 區分。

五、成果自評

- (1). LA-PCR :以 LA-PCR 法,我們順利完成四個 species 的 *groESL* 基因全長之 sequencing。此部份實驗並不容易。除了 DNA 品質要很好,也要有一點運氣。
- (2). 序列分析 :完成十個 species 的 *groESL* 基因之 sequencing。
- (3). 探討臨床菌株 *groESL* 基因變異情形,發現均有菌種內相當保守性及不同菌種間之差異性。
- (4) 探討 *groESL* 基因演化關係,提供菌種間演化關係另一參考。
- (5). 設計快速鑑定方法。

註:(1)至(4)的結果已發表於 2002 年 9 月 Journal of Clinical Microbiology 40 (9): 3172-3178.

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