行政院國家科學委員會專題研究計畫 成果報告

Stenotrophomonas maltophilia L1、L2 基因表現機制之探討及 L1、L2 蛋白受質特性分析

研究成果報告(精簡版)

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附件一

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

Stenotrophomonas maltophilia L1、L2 基因表現機制之探討及 L1、L2 蛋白受質特性分析

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共同主持人：
計畫參與人員：黃奕瑋, 黃紹晟, 林瑜姿, 林昕潔

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中　華　民　國　98　年　9　月　1　日

目　　錄

前言:

Stemptrphomonas maltophilia 在最近幾年已成為一重要的院內致病菌株。 因為其染色體可產生至少兩種 β-lactasmases (L1 及 L2)，所以使得 *S. maltophilia* 對大部分的 β-lactam 型的抗生素已產生抗藥性。目前為止，許多的研究報導都指出: L1 及 L2 基因的表現為誘發性，且 β-lactam 類型的抗生素為其誘發子。 最近，*S. maltophilia* K279a 的基因體定序工作正在進行中，由其部分已知的序列中發現: 位於 L2 基因上游，有一調控基因 *ampR*。類似 *S. maltophilia* L2 及其上游 *ampR* 基因的基因組裝曾在多種菌株的 AmpC β-lactamase 的調控系統中被報導。 *AmpC* 基因的調控方式已被研究較為清處，*ampC* 基因的表現受到三種調控蛋白的調控，分別為 AmpR, AmpG, AmpD。 在已知的 *S. maltophilia* K279a 的基因體序列中，可發現 *S. maltophilia* 亦有三個類似 *ampR*, *ampD* 及 *ampG* 的基因存在。如果能明白 L1、L2 基因的表現機制，則應可以調控蛋白作為治療策略的合適標的物。此成果報告主要為探討 AmpR 蛋白對菌體 L1 及 L2 β-lactamases 活性表現的調控角色。

研究目的:

S. maltophilia 的 L1 及 L2 基因的表現為誘發性 (inducible)，非持續性 (constitutive)，而 β-lactam 類的抗生素為其誘發子 (inducer)。 1989, Roster 及 Mett 的研究指出：利用單一的 β-lactam 當 inducer，L1 及 L2 可同時被誘發 (coinducibility)。 但在 Akova 等人的研究中曾報導一 *S. maltophilia* 突變株其有異常高值的 L2 活性，但 L1 的活性並無明顯上升；此現象似乎暗示著 L1 與 L2 活性的表現可能有不同的調控方式。 既然 L1、L2 基因為誘導表現型基因，所以可以推測菌體內應有些轉錄調控因子受到誘導因子 (inducer) 的誘導而表現，進而控制 L1、L2 因子的基因的表現。 如果能瞭解 L1 及 L2 如何受到 inducer 的誘導而表現其活性， 則可以阻止 L1 及 L2 的表現做為藥物設計的標的 (target)，抑制 L1 及 L2 的表現，再輔以一般的 β-lactam 治療，則有機會控制 *S. maltophilia* 的感染。

文獻探討:

參考文獻的報導發現，細菌體內的 β-lactamase 基因有兩種來源：一為內源性基因，亦即染色體上的基因 (chromosomal-mediated)； 一為外源性基因，如來自質體(plasmid) 或跳躍子 (transposon)。 *S. maltophilia* 內的 L1、L2 基因是屬於染色體上的基因。與 L1、L2 一樣同屬染色體基因的β-lactamases 亦在多種菌體中被報導，如 *Pseudomonas aeruginosa*、*Burkholderia cepacia*、*Morganella morganii*、*Hafnia alvei*、*Ochrobactrum anthropi*、*Serratia marcescens*、*Xanthomonas campestris*、 *Laribacter hongkongensis*。 分析文獻中此類菌體的 β-lactamases 基因常具有以下特徵：(1) 若其活性的表現為誘導式(inducible)，則此菌體內有一 *ampR* 基因，且此基因與誘發性 β-lactamase 活性有關。 (2) 若其活性的表現為持續型 (constitutive)，則此菌體內無 *ampR* 基因。 (3)若此菌體有 *ampR* 基因，其 *ampR* 基因常位於 β-lactamase 基因之上游，但 *ampR* 基因走向與 β-lacamase 基因方向相反。此種 AmpR-β-lactamase 基因組成的調控組裝在很多革蘭氏陰性菌中被發表，但依其特徵的差異有兩種不同的型式。

<u>第一種型式為 AmpR-AmpC 調控組裝</u>，可見於 *Citrobacter freundii* OS60、*Enterobacter cloacae* 55、*Acinetobacter*、*Pseudomonas aeruginosa*。 此型的 β-lactamase (AmpC) 屬於 Ambler's 分類 class C，Bush's 分類 group 1 型。 此型的調控機制被研究的最清楚，共有四個基因參與 induction 的調控，分別為 *ampD*, *ampE*, *ampG* 及 *ampR*。其主要機制分述如下: (1) 當沒有 β-lactam 抗生素時 (no inducer)，*AmpR* 基因會 constitutive expreesion，所表現的 AmpR 蛋白會結合在 *ampR* 與 *ampC* 基因的 intergenic region，而影響 *ampC* 表現，所以在沒有 inducer 時，AmpR 為一 repressor，抑制 *ampC* 表現 (菌體沒有 β-lactamase 活性)。 (2) 當 β-lactam 型的抗生素(inducer) 進入細菌之 periplasma 中時，會抑制 periplasma 中的 UDP-MurNAc-pentapeptide 合成 murein，所以過多的 UDP-MurNAc-pentapeptide 在 periplasma 中被分解成 1,6-anhydromuropeptides。 AmpG 為一 transmembrane protein，其可將 1,6-anhydromuropeptides 送至 cytosol 中。1,6-anhydromuropeptides 為活化 AmpR 的重要 ligand，其可改變 AmpR repressed condition (relieve the repressed state of AmpR)，而使得 *ampC*

基因可表現，菌體表現 β-lactamase 活性。所以 AmpG 對菌體 β-lactamase 活性的 induction 為一 positive regulator。

(3) AmpD 為一 cytosol protein，其為一 N-acetyl-anhydromuramyl-L-alanine amidase，其可水解 1,6-anhydromuropeptides，使得 induction 不發生，所以 AmpD 對菌體 β-lactamase 活性的 induction 為一 negative regulator。

(4) AmpE 為一 tansmembrane protein，在諸多菌體中，*ampE* 與 *ampD* 形成一 operon。但依目前的研究報導，AmpE 與 β-lactamase 的 induction 似乎沒有具體的相關性。

第二種型式為 AmpR-Class A β-lactamase 調控組裝。此計畫所研究的菌體 *S. maltophilia* 之 L2 基因即屬 AmpR-ClassA β-lactamase 調控組裝。目前已有兩株 *S. maltophilia* 菌株 (R551-3 及 K279a) 的基因體序列可供參考。基因體序列搜索的結果發現，*S. maltophilia* 菌體中有一個 *ampR* homologue，兩個 *ampD* homologues 及一個 *ampG* 基因。此型的調控機制尚未被研究得很清楚。

研究方法:

(A) 不同菌種間 AmpR-β-lactamase 調控組裝之演化分析

(1) 從 NCBI 資料庫中搜索不同菌種具有 *ampR*-β-lactamase 組裝的 DNA 序列

(2) 以 bioinformatics 分別分析不同菌種之 AmpR, IG region 及 L2 之相關性

(B) AmpR 對 L1 β-lactamase 基因表現所扮演的角色

(1) 構築 *S. maltophilia* KH 之 KHΔL2 mutant 及 KHΔRL2 之 double mutant。

(2) 比較 KHΔL2 及 KHΔRL2 在有 inducer 及無 inducer 情形下所表現的 β-lactamase 活性之比較分析

(C) AmpR 對 L2 β-lactamase 基因表現所扮演的角色

(1) 構築 *S. maltophilia* KH 之 KHΔR 及 KHΔL1 mutants

(2) 比較 KHΔR 及 KHΔL1 在有 inducer 及無 inducer 情形下所表現的 β-lactamase 活性之比較分析

(D) AmpR autoregulation 的評估

可由下列實驗的設計，瞭解 AmpR 是否有 autoregulation 的現象

① KH(pKHR$_{xylE}$174L2)及 KHΔRL2(pKHR$_{xylE}$174L2) 在有 inducer 及無 inducer 情形下所表現的 C23O 活性之比較分析

結果與討論:

(A) 不同菌種間 AmpR-β-lactamase 調控組裝之演化分析

共搜索 9 株 *ampR-ampC* module 菌株及 8 株 *ampR-class A β-lactamase* module 菌株。其 β-lactamase 蛋白之演化分析如圖 1(A), AmpR 蛋白之演化分析如圖 1(B), *ampR-β-lactamase* intergenic region (IG) 之比對分析呈現於圖 2。

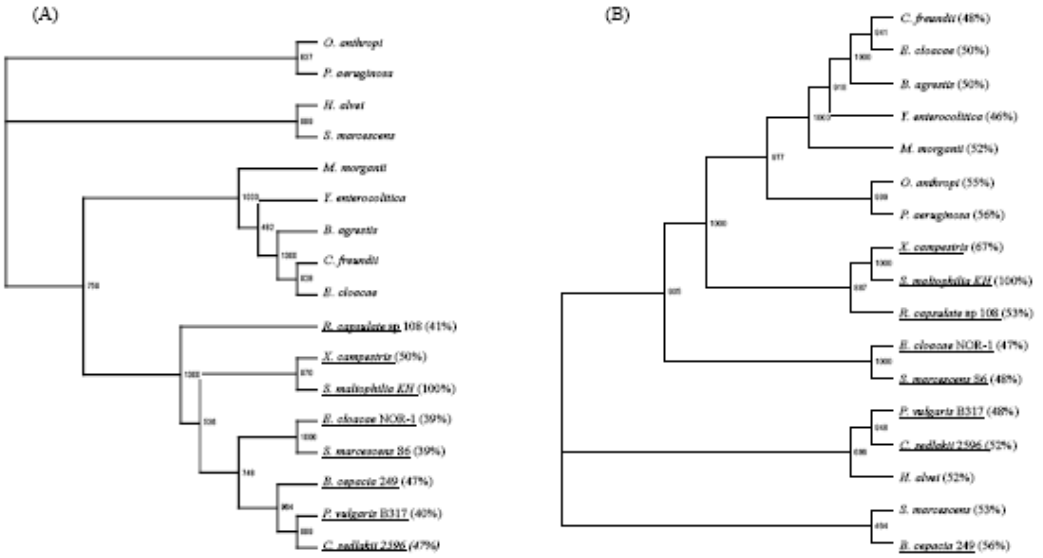

Fig. 1. Dendrograms showing the relationships of L2 and AmpR proteins of *S. maltophilia* KH to the related β-lactamases and AmpR proteins. The dendrogram is constructed by using the amino acid sequences from the assayed proteins. The bootstrap numbers at the branch points refer to 1000 replications. The species with a class A β-lactamase are underlined and the other are the type of AmpC β-lactamases. (A) Dendrogram of chromosomally encoded class C and class A β-lactamases. The number in each bracket denotes the identity between the indicated β-lactamase and L2 of *S. maltophilia* KH. (B) Dendrogram of chromosomally encoded AmpR proteins. The number in each bracket denotes the identity between the indicated AmpR and AmpR of *S. maltophilia* KH.

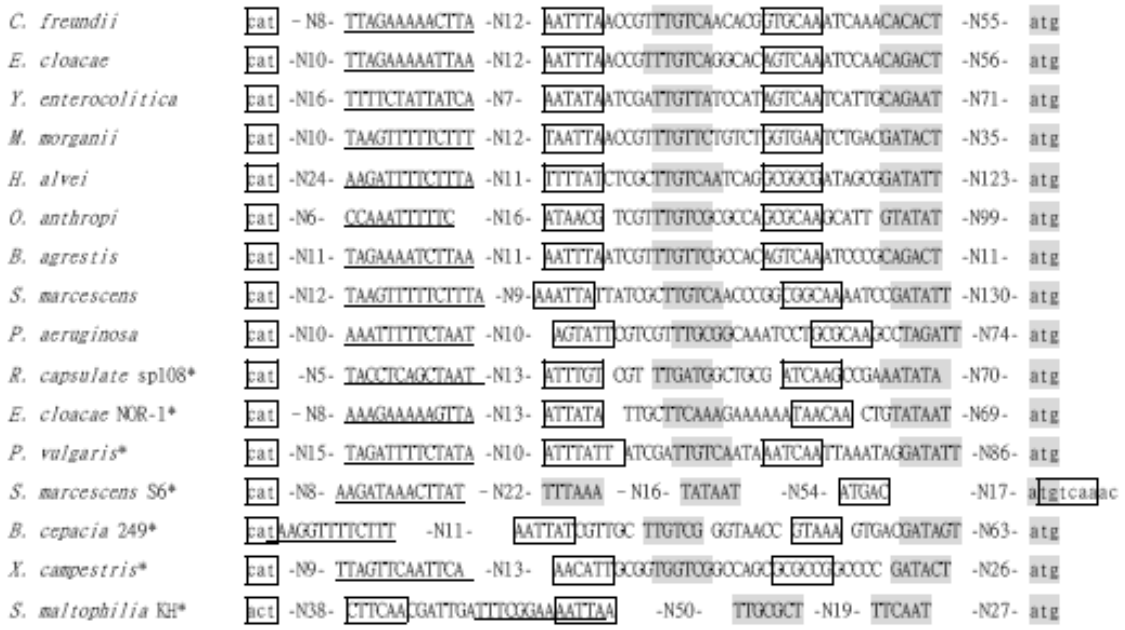

Fig. 2. Alignment of the *ampR-β-lactamase* IGs with that of *S. maltophilia* KH. The start codon and promoters (-10 and -35 regions) for *ampR* genes are boxed, and those for L2 genes are shaded. The putative LysR binding motif for AmpR binding is underlined. The strains marked with asterisks indicate the type of *ampR-class A β-lactamase* module and the other strains are the type of *ampR-ampC* system.

(B) AmpR 對 L1 β-lactamase 基因表現所扮演的角色

分析 KHΔL2 及 KHΔRL2 在有 inducer 及無 inducer 情形下所表現的 β-lactamase 活性 (表一)

Table 1. The β-lactamase activity and C23O activity of *E. coli* and *S. maltophilia* harboring different recombinant plasmids

Strain(plasmid)	Genotype Strain/plasmid	β-lactamase activity (Un[a]/mg)		C23O activity (Uc[b]/mg)	
		Uninduced	Induced	Uninduced	Induced
E. coli					
DH5α(pKHR174L2)	Wild type / *ampR*$^+$, *L2*$^+$	0	0	-	-
DH5α(pKHR174L2$_{xylE}$)	Wild type / *ampR*$^+$, *L2*::*xylE*	-	-	0	0
S. maltophilia					
KH(pKHR174L2$_{xylE}$)	Wild type / *ampR*$^+$, *L2*::*xylE*	-	-	2	52
KHΔL2	*ampR*$^+$, *L1*$^+$, *L2*$^-$	2	136	-	-
KHΔRL2	*ampR*$^-$, *L1*$^+$, *L2*$^-$	0	0	-	-
KHΔR	*ampR*$^-$, *L1*$^+$, *L2*$^+$	12	12	-	-
KHΔL1	*ampR*$^+$, *L1*$^-$, *L2*$^+$	4	382	-	-
KH(pKHR$_{xylE}$174L2)	Wild type / *ampR*::*xylE*, *L2*$^+$	-	-	4.1	4.0
KHΔRL2(pKHR$_{xylE}$174L2)	*ampR*$^-$, *L1*$^+$, *L2*$^-$ / *ampR*::*xylE*, *L2*$^+$	-	-	4.0	4.2

所以, AmpR 對 L1 β-lactamase 基因表現所扮演的角色: 不論在有無 inducer 的情況下, AmpR 對 L1 基因的表現為正調控。

(C) AmpR 對 L2 β-lactamase 基因表現所扮演的角色

分析 KHΔR 及 KHΔL1 在有 inducer 及無 inducer 情形下所表現的 β-lactamase 活性 (表一)

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KHΔL1	*ampR*$^+$, *L1*$^-$, *L2*$^+$	4	382	-	-
KH(pKHR$_{xylE}$174L2)	Wild type / *ampR*::*xylE*, *L2*$^+$	-	-	4.1	4.0
KHΔRL2(pKHR$_{xylE}$174L2)	*ampR*$^-$, *L1*$^+$, *L2*$^-$ / *ampR*::*xylE*, *L2*$^+$	-	-	4.0	4.2

所以, AmpR 對 L2 β-lactamase 基因表現所扮演的角色: 不論在無 inducer 的情況下, AmpR 對 L2 基因的表現為負調控。在有 inducer 的情況下, AmpR 對 L2 基因的表現為正調控。

(D) AmpR autoregulation 的評估

分析 KH(pKHR $_{xylE}$174L2)及 KHΔRL2(pKHR $_{xylE}$174L2) 在有 inducer 及無 inducer 情形下所表現的 C23O 活性之比較分析 (表一)。

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DH5α(pKHR174L2)	Wild type / *ampR*$^+$, *L2*$^+$	0	0	-	-
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S. maltophilia					
KH(pKHR174L2$_{xylE}$)	Wild type / *ampR*$^+$, *L2*::*xylE*	-	-	2	52
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KHΔRL2(pKHR$_{xylE}$174L2)	*ampR*$^-$, *L1*$^+$, *L2*$^-$ / *ampR*::*xylE*, *L2*$^+$	-	-	4.0	4.2

所以, AmpR 沒有 autoregulation 的現象。

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Cheng-Wen Lin, Yi-Wei Huang, Rouh-Mei Hu, Kai-Hung Chiang, **Tsuey-Ching Yang***. 2009. The role of AmpR in the regulation of L1 and L2 β-lactamases in *Stenotrophomonas maltophilia*. **Research in Microbiology**. **160:**152-158.

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Stenotrophomonas maltophilia 的 ampR-L2 組裝之特性分析。黃奕瑋。中國醫藥大學醫學檢驗生物技術學系碩士班。2009,1 月。

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The role of AmpR in regulation of L1 and L2 β-lactamases in *Stenotrophomonas maltophilia*

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Abstract

Stenotrophomonas maltophilia is known to produce at least two chromosomal-mediated inducible β-lactamases, L1 and L2. Gene *L2*, which encodes a class A β-lactamase, and the adjacent *ampR* gene form an *ampR*-class A β-lactamase module. L1 belongs to the class B β-lactamase and has no neighbor *ampR*-like regulatory gene. In this study, the *ampR-L2* module from *S. maltophilia* KH was compared with *ampR*-β-lactamase modules from several microorganisms with respect to the AmpR and β-lactamase proteins and the intergenic (IG) region. *S. maltophilia* and *Xanthomonas campestris* showed the most closely phylogenetic relationship among the microorganisms considered. The regulatory role of AmpR towards L1 and L2 was further analyzed. In the absence of an inducer, AmpR acted as an activator for *L1* expression and as a repressor for *L2* expression, whereas AmpR was an activator for both genes in an induced state. In addition, inducibility of *L1* and *L2* genes depended on the presence of AmpR. The *ampR* transcript was weakly and constitutively expressed, but was not autoregulated.

Keywords: *Stenotrophomonas maltophilia*; β-lactamase; AmpR

1. Introduction

β-lactamase production is a major mechanism causing bacterial resistance to β-lactam antibiotics [1]. The genomic module of *ampR* and an inducible β-lactamase gene has been commonly seen in many gram-negative bacteria, in which both genes are divergently transcribed and the intergenic region (IG) between them contains the promoters and the regulatory domains [12]. Two different classes of β-lactamases have been identified in such an *ampR*-β-lactamase module, i.e. AmpC and class A β-lactamase.

Chromosomally encoded *ampR-ampC* systems have been found in *Citrobacter freundii* [18], *Enterobacter cloacae* [13], *Yersinia enterocolitica* [40], *Morganella morganii* [33], *Hafnia alvei* [11], *Ochrobactrum anthropi* [25], *Buttiauxella agrestis* [5], *Serratia marcescens* [22] and *Pseudomonas aeruginosa* [21]. The regulation of chromosomal *ampR-ampC* systems is well documented [15,19,29,44]. In *C. freundii*, induction of *ampR-ampC* is intimately linked to peptidoglycan recycling. There are at least three gene products known to be involved in the induction mechanism: AmpG, AmpD and AmpR. AmpG encodes a transmembrane protein that functions as a permease to transport the cell wall degradation products such as anhMurNAc-tripeptide from the periplasm into the cytoplasm. AmpD encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase, which hydrolyzes the anhMurNAc-tripeptide for further recycling. The *ampR* gene encodes an LysR-type transcriptional regulator (LTTR), which controls *ampC* gene transcription. The *ampR-ampC* IG region of *C. freundii* has been characterized. The promoters for *ampR* and *ampC* are located in the IG region in opposite orientation and partially overlap. A consensus T-N11-A LysR-motif is conserved and located in the *ampR* promoter region, resulting in the negative autoregulation of *ampR* [20]. Similar IG architecture is observed in many other *ampR-ampC* modules. Furthermore, AmpR acts as a repressor in

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the absence and as an activator in the presence of the β-lactams [15,18,19]. In addition to the chromosomally encoded *ampR-ampC* systems, many plasmid-born AmpC β-lactamases have been discovered in bacteria [4,6,10,26,31,32], including some clinical isolates [3,9,27,35,42].

Another similar regulatory module, *ampR*-class A β-lactamase, has been identified in *Rhodopseudomonas capsulate* sp108 [7], *E. cloacae* NOR-1 [23], *Proteus vulgaris* B317 [8], *Serratia marcescens* S6 [24], *Burkholderia cepacia* 249 [41], *Citrobacter sedlakii* 2596 [37], *Xanthomonas campestris* [43] and *Stenotrophomonas maltophilia* [14]. The *ampR*-class A β-lactamase modules described so far are all chromosomal. Compared to the *ampR-ampC* module, the IG region architectures of *ampR*-class A β-lactamase modules are more diverse. For example, in *S. marcescens* S6, the promoters of the *ampR* and class A β-lactamase genes exhibit a face-to-face architecture [24]. In addition, the regulatory role of AmpR proteins is different in the different *ampR*-class A β-lactamase modules. AmpR acts as an activator regardless of the presence of an inducer in *E. cloacae* NOR-1 [23] and *S. marcescens* S6 [24], but it acts as a repressor in *B. cepacia* 249 [41].

S. maltophilia, a non-fermenting gram-negative bacillus, is known to produce at least two chromosomally encoded inducible β-lactamases, L1 and L2 [2,36,37]. The corresponding genes *L1* and *L2* and their flanking DNA sequences have been separately cloned and analyzed in our previous study [14]. Recently, AmpR was shown to be a key regulator for inducible expression of *L1* and *L2* [30]. This work aims at identifying the regulatory role of AmpR towards basal *L1* and *L2* gene expression and at testing the autoregulation of AmpR expression. Moreover, the *ampR-ampC* and *ampR*-class A β-lactamase systems are compared and discussed in detail.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Table 1 shows the bacterial strains and plasmids used in this study. *S. maltophilia* KH is a clinical isolate. The active β-lactamases present in *S. maltophilia* KH are encoded by the *L1* and *L2* genes, as verified by isoelectric focusing electrophoresis after nitrocefin staining (our unpublished data). *Escherichia coli* DH5α and *E. coli* S17-1 were used for general cloning and conjugation experiments, respectively. For the induction experiment, 10 μg/ml of cefuroxime was added.

2.2. Construction of recombinant plasmids pKHR174L2$_{xylE}$, pKHΔRL2, pKHΔL2, pKHΔR, pKHR$_{xylE}$174L2, and pKHΔL1

Fig. 1A shows the restriction enzyme map of the *S. maltophilia* KH *ampR-L2* module (accession No. EU032534). A 1.3-bp *xylE* cassette derived from pTXylE [14] was inserted into the SmaI site in the *ampR* gene and in the SphI site of *L2* in pKHR174L2. The resultant recombinant plasmids, pKHR$_{xylE}$174L2 and pKHR174L2$_{xylE}$, were sequenced to confirm the correct orientation of *xylE* gene. The recombinant plasmids pKHΔRL2 and pKHΔR were derived from pKHR174L2 by deleting a 797-bp StuI and a 468-bp PstI fragment, respectively. The 608-bp SmaI-StuI fragment from pKHR174L2 was ligated into the SmaI-digested pEX18Tc, and the resultant plasmid was further ligated with the 854-bp SphI-HindIII fragment from pKHR174L2, producing pKHΔL2. The correctness of both inserted DNA fragments in pKHΔL2 was checked by DNA sequencing. The intact *L1* gene of *S. maltophilia* KH was amplified by PCR using the paired primers 5′-AAGGAG GCC CAT GCT AGT TT-3′ and 5′-TTC TGA CCG GCA CCC TTC-3′, and cloned into the T-vector (pTKHL1). The recombinant plasmid pKHΔL1 was constructed by a two-step cut-ligation of HindIII-PstI and XbaI-SphI from pTKHL1 to pEX18Tc treated with the same restriction enzymes. Again, DNA sequencing was performed to check the correctness of both insertions.

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or properties
Stenotrophomonas maltophilia	
KH	Wild type, a clinical isolate from Taiwan
KHΔL1	*S. maltophilia* KH isogenic mutant of *L1* gene
KHΔL2	*S. maltophilia* KH isogenic mutant of *L2* gene
KHΔRL2	*S. maltophilia* KH double mutant of *L2* and *ampR* gene; deletion 797 bp StuI-StuI DNA fragment which including partial 5′ terminus of *ampR*, intergenic region of *ampR* and L2, and partial 5′ terminus of *L2*
Escherichia coli	
DH5α	F- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44*χ *thi-1 gyrA96 relA1*
S17-1	λ pir + mating strain
Plasmids	
pEX18Tc	*sacB oriT*, Tc^r
pKHR174L2	pEX18Tc vector with a 2.8 kb PCR amplicon containing the partial 3′ terminus of ABC transport protein gene, *ampR* gene, 174-bp IG region, and *L2* gene; Tc^r
pKHR174L2$_{xylE}$	Derived from pKHR174L2, inserting an *xylE* cassette of the same orientation as *L2* gene into SphI site of *ampR* gene; Tc^r
pKHΔL1	pEX18Tc vector with a 298-bp XbaI-SphI DNA fragment of 5′ terminus of *L1* gene and a 336-bp PstI-HindIII DNA fragment of 3′ terminus of *L1* gene, deleting the internal 407-bp DNA fragment of *L1* gene
pKHΔL2	Derived from pKHR174L2, deleting the 107-bp StuI-SphI DNAa fragment; Tc^r
pKHΔR	Derived from pKHR174L2, deleting the 468-bp PstI-PstI DNA fragment; Tc^r
pKHΔRL2	Derived from pKHR174L2, deleting the 797-bp StuI-StuI DNA fragment; Tc^r
pKHR$_{xylE}$174L2	Derived from pKHR174L2, inserting an *xylE* cassette of the same orientation as *ampR* gene into the SmaI site of *ampR* gene; Tc^r

2.3. Construction of isogenic mutants KHΔL2, KHΔRL2, KHΔR, and KHΔL1

Recombinant plasmids pKHΔL2, pKHΔRL2, pKHΔR, and pKHΔL1 were introduced into *S. maltophilia* KH via conjugation to generate isogenic mutants KHΔL2, KHΔRL2,

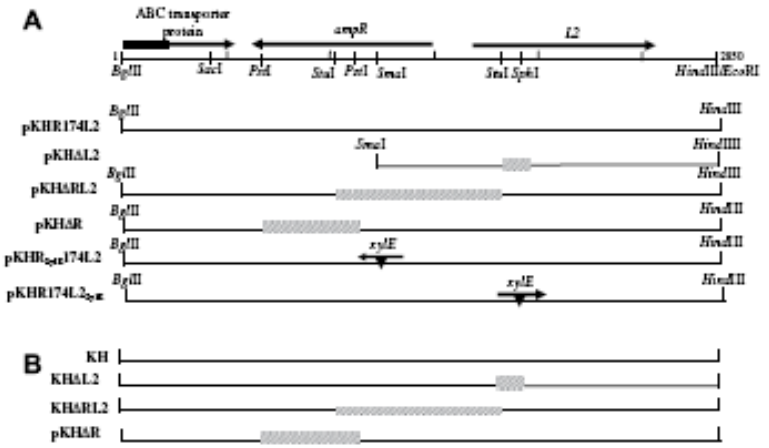

Fig. 1. Restriction enzyme map of a segment of the *S. maltophilia* KH chromosome surrounding the *ampR-L2* module. (A) DNA inserts of plasmids pKHR174L2, pKHR174L2$_{xylE}$, pKHΔL2, pKHΔRL2, pKHΔR and pKHR$_{xylE}$174L2. All plasmids are derivatives of pEX18Tc. The cross-hatched box indicates the deleted region. The inserted site of the *xylE* cassette is marked by (▼) and the direction of *xylE* gene is indicated by the arrow. (B) The genomic organization of wild strain KH and its isogenic mutants KHΔL2, KHΔRL2 and KHΔR.

KHΔR, and KHΔL1 by homologous recombination as described previously [14,39]. The correctness of the mutants was checked by PCR and sequencing (Fig. 1B).

2.4. β-lactamase activity assay

Bacterial cultures were prepared as described [14], except that 10 μg/ml of cefuroxime were added as the inducer. β-lactamase activity was determined spectrophotometically using hydrolysis of nitrocefin (Oxoid, UK) in 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM $ZnCl_2$. The specific activity (U/mg) was expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein, using a Δε of 20,500 M^{-1} cm^{-1} for nitrocefin at 486 nm as suggested by the manufacturer (Oxoid, UK). Protein concentration was determined using the Bio-Rad protein assay reagent, with bovine serum albumin as a standard.

2.5. Catechol 2,3-dioxygenase (C23O) activity assay

C23O activity was measured in intact cells [16]. Activity assays were performed in a buffer with 50 mM sodium phosphate buffer, 10% acetone, and 0.1 M catechol as the substrate. Hydrolysis of catechol was examined by spectrophotometric analysis at a wavelength of 375 nm. The rate of hydrolysis was calculated by using 44,000 M^{-1} cm^{-1} as the extinction coefficient. One unit of enzyme activity (Uc) was defined as the amount of enzyme that converts 1 nmole substrate per min. The specific activity (Uc/OD_{450nm}) of the enzyme was defined in terms of units per 3.6×10^8 cells (assuming that an A_{450nm} of 1 corresponds to 3.6×10^8 cells/ml).

2.6. Bioinformatics analysis

The homologues of AmpR and L2 proteins were searched by the BLASTP program available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov), using the AmpR and L2 protein sequences of *S. maltophilia* KH as the query. The IG region DNA sequences of the different modules were then obtained from the indicated PubMed references. Multiple sequence alignments among the assayed DNA and proteins were performed using the ClustalX program. Phylogenetic trees were constructed using the neighbor-joining methods. The bootstrap number was obtained in 1000 replications.

2.7. Nucleotide sequence accession number

The nucleotide sequences of *S. maltophilia* KH *ampR-L2* module and *L1* gene have been deposited in GenBank under accession no. EU032534 and EU441218.

3. Results

3.1. Molecular phylogenetic analysis of the ampR-β-lactamase modules

Characteristic elements of Amber class A β-lactamase have been identified in the L2 protein of *S. maltophilia* KH, showing that the *ampR*-L2 module is the member of the *ampR*-class A β-lactamase family [36]. The *ampR-L2* module was compared with nine *ampR-ampC* modules and seven *ampR*-class A β-lactamase modules from different species. The similarity dendrograms of β-lactamase and AmpR are shown in Fig. 2.

Two obvious clusters of *ampC* and class A β-lactamases were found in the dendrogram. The L2 β-lactamase of *S. maltophilia* KH was 39–50% identical to those of *ampR*-class A β-lactamase modules, but showed insignificant similarity with *ampR-ampC* systems (Fig. 2A).

S. maltophilia KH AmpR displayed 46–67% identity to the known AmpR proteins (Fig. 2B). Conserved amino acids

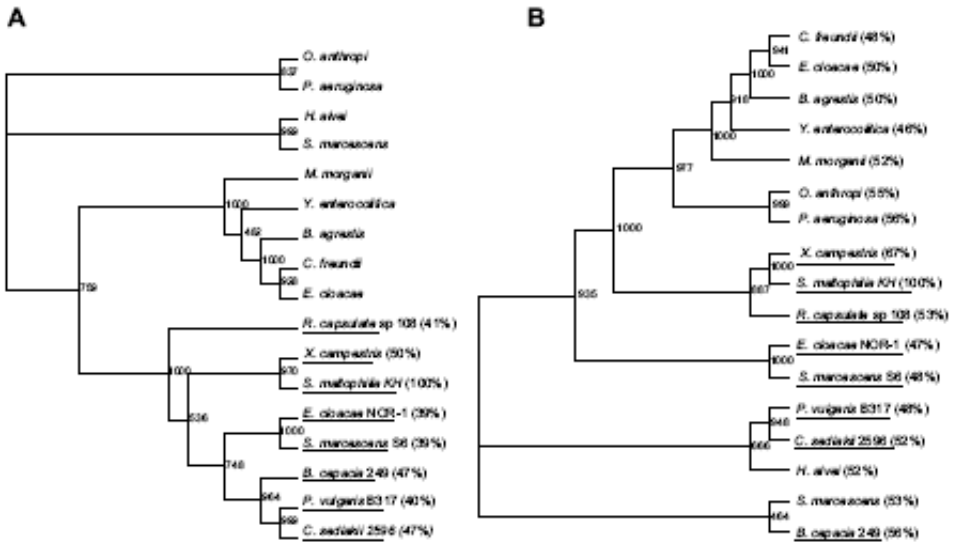

Fig. 2. Dendrograms showing the relationship of L2 and AmpR proteins of *S. maltophilia* KH to related β-lactamases and AmpR proteins. The dendrogram was constructed using the amino acid sequence of the proteins. The bootstrap numbers at the branch points refer to 1000 replications. Species harboring class A β-lactamases are underlined. The others carry AmpC β-lactamases. (A) Dendrogram of chromosomally encoded class C and class A β-lactamases. The numbers in brackets denote percentage of identity between the indicated β-lactamase and *S. maltophilia* KH L2. (B) Dendrogram of chromosomally encoded AmpR proteins. The numbers in brackets denote percentage of identity between the indicated AmpR and AmpR of *S. maltophilia* KH.

among the AmpR proteins aligned were randomly distributed without obvious clusters (data not shown). Furthermore, a helix-turn-helix motif was found at the N-terminal end of all assayed AmpR proteins.

The highest levels of identity to *S. maltophilia* KH AmpR and L2 were found with those of *X. campestris* (identity of 67 and 50%, respectively). Noteworthy among the microorganisms considered, the dendrogram of AmpR was not always parallel to that of β-lactamases. The AmpR of *S. maltophilia* KH was more closely related to AmpR proteins within some *ampR-ampC* systems (e.g. *P. aeruginosa* and *O. anthropi*) than to those in *ampR*-class A β-lactamase modules (*P. vulgaris* B317 and *E. cloacae* NOR-1). On the contrary, *S. maltophilia* KH L2 shared higher similarity with *ampR*-class A β-lactamases (Fig. 2B).

Fig. 3 shows the alignment of *ampR*-β-lactamase IG regions from *S. maltophilia* KH and 15 other *ampR*-β-lactamase systems. The length of IG region ranged from 86 to 212 nucleotides. Structural features typical of the *ampR*-β-lactamase IG were more conserved in the *ampR-ampC* systems than in the *ampR*-class A β-lactamases. The 174-bp IG of *S. maltophilia* KH exhibited specific organization with no significant similarity with the other sequences. Potential −10 and −35 consensus sequences of the *ampR* and *L2* gene promoters were predicted (http://www.fruitfly.org/) as shown in Fig. 3, with a back to back arrangement separated by 50 nucleotides. The LysR binding motif T-N11-A is underlined in Fig. 3. A putative AmpR binding region, TTTTTGCGCTGGT, was found to overlap the predicted −35 region of the *S. maltophilia* KH *L2* gene promoter.

3.2. Expression of the *ampR-L2* module of S. maltophilia KH in an E. coli system

Most assays of *ampR-ampC* or *ampR*-class A β-lactamase modules use *E. coli* as a host for expression. Unfortunately, when transformed in *E. coli*, the *ampR-L2*-containing plasmid pKHR174L2 did not express detectable β-lactamase activity (data not shown). Consistent with this result, plasmid pKHR174L2$_{xylE}$ in which the *L2* gene was replaced by an *xylE* gene expressed C23O activity in the presence of an inducer in *S. maltophilia* KH but not in *E. coli* (data not shown).

3.3. The role of AmpR in L1 expression

Since *E. coli* was not a suitable system for the assay of *ampR-L2*, a series of isogenic mutants of *S. maltophilia* KH, KHΔL2, KHΔRL2, KHΔR, and KHΔL1 were constructed. To elucidate the role of AmpR in the expression of *L1*, β-lactamase activity was measured in strains KHΔL2 and KHΔRL2. As illustrated in Table 2, induced KHΔL2 displayed higher β-lactamase activity (136 U/mg) than the uninduced counterpart (2 U/mg); KHΔRL2, however, had lost β-lactamase activity (0 U/mg) in both basal and induced conditions. AmpR is thus required for both the basal level and the induced expression of L1 β-lactamase.

3.4. The role of AmpR in L2 expression

To further elucidate the role of AmpR in expression of *L2*, β-lactamase activity was measured in KHΔR and KHΔL1

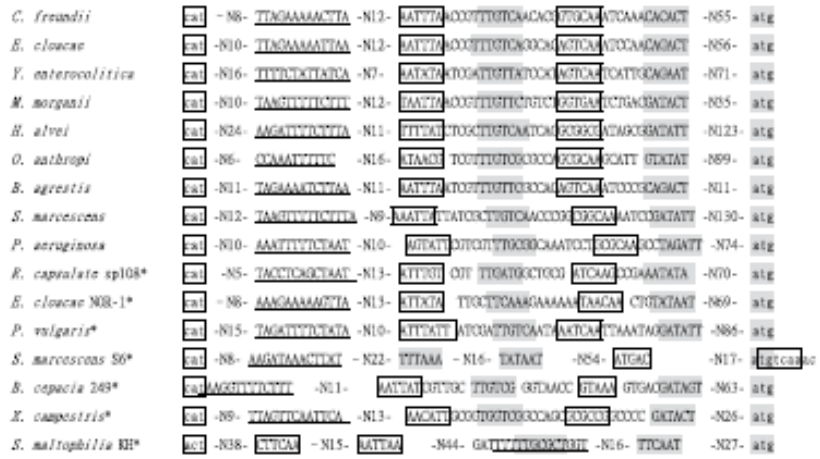

Fig. 3. Alignment of *ampR*-β-lactamase intergenic regions (IGs) with that of *S. maltophilia* KH. The start codon and promoter (−10 and −35 regions) for the *ampR* genes are boxed, and those for *L2* genes are shaded. The putative AmpR binding motif is underlined. Strains marked with asterisks carry *ampR*-class A β-lactamase modules. The other strains carry *ampR-ampC* systems.

(Table 2). In the induced KHΔRL2, the absence of AmpR resulted in a complete loss of L1 activity, such that KHΔR appeared suitable for evaluating L2 activity in *ampR*$^-$ conditions. β-lactamase activity in KHΔR and KHΔL1 was 12 and 4 Un/mg in the absence of an inducer and 12 and 382 U/mg after induction. AmpR thus acts as a weak repressor of *L2* expression in the absence of an inducer and as an activator in the induced state. Basal constitutive β-lactamase activity (12 Un/mg) was detected in extracts of KHΔR, indicating that *L2* basal expression is AmpR-independent and that induction of *L1* and *L2* genes is AmpR-dependent.

3.5. Evaluation of AmpR autoregulation

LysR-type regulators are notably autoregulated [38]. Hence, autoregulation of *ampR* in *S. maltophilia* was tested. Recombinant plasmid pKHR$_{xylE}$174L2 was introduced in strains KH and KHΔRL2, which provide conditions with and without AmpR proteins, respectively. Table 2 shows that the C23O activity in KH/pKHR$_{xylE}$174L2 and KHΔRL2/pKHR$_{xylE}$174L2 was relatively low and did not change significantly upon addition of an inducer or the presence of AmpR. The expression of *ampR* was thus weak and constitutive as well as was inducer- and AmpR-independent.

4. Discussion

AmpR is a regulator that simultaneously controls induction of *L1* and *L2* β-lactamase genes [30]. Nevertheless, *L1* and *L2* genes seem to be differently regulated. (1) AmpR is essential and acts as an activator for basal level expression of the L1 enzyme. In contrast, basal level expression of L2 is AmpR-independent and negatively regulated by AmpR (Table 2). (2) A conserved LysR binding motif sequence [38] could be predicted in the *L2* but not in the *L1* upstream region. These results are consistent with *S. maltophilia* KH *L2* being regulated by AmpR as in the *ampR-ampC* system [17,29,44], i.e. as a weak repressor without an inducer, but as an activator with

Table 2
The β-lactamase activity and C23O activity of *E. coli* and *S. maltophilia* harboring different recombinant plasmids

Stain (plasmid)	Genotype stain/plasmid	β-lactamase activity (Un[a]/mg)		C23O activity (U[b]/OD$_{650\ nm}$)	
		Uninduced	Induced	Uninduced	Induced
S. maltophilia					
KH(pKHR174L2$_{xylE}$)	Wild type/*ampR*$^+$, *L2*::*xylE*	–	–	2	52
KHΔL2	*ampR*$^+$, *L1*$^+$, *L2*$^-$	2	136	–	–
KHΔR12	*ampR*$^-$, *L1*$^+$, *L2*$^-$	0	0	–	–
KHΔR	*ampR*$^-$, *L1*$^+$, *L2*$^+$	12	12	–	–
KHΔL1	*ampR*$^+$, *L1*$^-$, *L2*$^+$	4	382	–	–
KH(pKHR$_{xylE}$174L2)	Wild type/*ampR*::*xylE*, *L2*$^+$	–	–	4.1	4.0
KHΔR12(pKHR$_{xylE}$174L2)	*ampR*$^-$, *L1*$^+$, *L2*$^-$/*ampR*::*xylE*, *L2*$^+$	–	–	4.0	4.2

[a] One unit of β-lactamase is defined as 1 nanomole of nitrocefin hydrolyzed per minute per mg protein. Results are geometric means of three independent determinations. Standard derivations were within 10% of the geometric means in all cases.

[b] One unit of catechol 2,3-dioxygenase is defined as 1 nanomole of catechol hydrolyzed per minute per 3.6×10^8 cells. Results are geometric means of three independent determinations. Standard derivations were within 10% of the geometric in all cases.

an inducer [13,17,18,33]. In the case of *L1*, AmpR always acts as a positive regulator, which is similar to the situation for some *ampR*-class A β-lactamase systems [23,24,43].

ampR-ampC regulation is usually analyzed using *E. coli* as a heterologous experimental system [13,18,19]. Such studies lead to the conclusion that *E. coli* AmpG and AmpD may act in trans for inducible expression of cloned *ampC* in the presence of *ampR*. Similar experimental strategies also work for *ampR*-class A β-lactamase modules [8,23,24,28,34,41], except for that of *X. campestris* [43]. Here we show that the *S. maltophilia* KH *ampR-L2* module cannot be expressed in *E. coli*. This is consistent with the close relationship we observed between *S. maltophilia* KH *ampR-L2* and that of *X. campestris* [43].

Autoregulation of AmpR has been observed in *C. freundii* [20] and *S. marcescens* S6 [24] as a threefold decrease in *ampR* expression in the presence of the AmpR protein. On the contrary, in *P. aeruginosa*, *ampR* is not autoregulated [17]. In this study, we show that this is also the case for *S. maltophilia ampR*.

Many chromosomally encoded β-lactamase genes can coexist in a given microorganism [14,17,23,24,28,34]. In some, each β-lactamase is within an independent *ampR*-β-lactamase module [23,24,28,34] and individual β-lactamases are regulated by their cognate *ampR* gene. However, in *P. aeruginosa*, the *ampR* gene regulates the contiguous *ampC* gene as well as the unlinked *poxB* gene [17]. The case of *S. maltophilia* would thus resemble that of *P. aeruginosa*, since the *L1* and *L2* genes are both regulated by the AmpR protein [30]. While in *P. aeruginosa*, AmpR acts on as an activator for the *ampC* gene but as a repressor on *poxB* [17], in *S. maltophilia* AmpR acts as a positive regulator of *L1* and *L2* in the presence of inducers. Therefore, AmpR appears as a potential target for antimicrobials that would block expression of the *ampR* gene or the function of the AmpR protein to overcome the β-lactam resistance of *S. maltophilia*.

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計畫成果自評:

本計畫執行情形順利。除了其中幾個突變株的構築改變策略外，其他大部份皆依照計畫書所設計內容執行。成果已發表於學術性期刊 Research in Microbiology.