จากรูป 12 เมื่อเปรียบเทียบแถบขนาดของ PCR product ใน Lane ที่ 2 และ 3 กับ DNA marker ใน Lane ที่ 1 พบว่า PCR product ในหลุมที่ 2 และ3 มีขนาดใกล้เคียงกับขนาดของ DNA ประมาณ 1 Kb แปลผลได้ว่า PCR product จากเชื้อ *B. pseudomallei* สายพันธุ์ 1902a (Lane 2) และ สายพันธุ์ E15 (Lane 3) มีขนาดใกล้เคียงกับ class B β-lactamase-like gene

13. การหาลำดับเบสของ class B β-lactamase-like gene จากเชื้อ *B. pseudomallei*

เนื่องจาก PCR product ที่ได้จากเชื้อ *B. pseudomallei* สายพันธุ์ 1902a และ E15 มีขนาดใกล้เคียงกับ PCR product ที่ต้องการ ดังนั้นเพื่อเป็นการตรวจสอบว่าเป็น class B β-lactamase-like gene จึงทำการหาลำดับเบสของ PCR product โดยการทำ PCR product ให้บริสุทธิ์แล้วส่งไปหาลำดับเบสที่หน่วยบริการชีวภาพ (BioService Unit) ผลที่ได้แสดงในรูป 13

Open reading frame นี้มีความยาวทั้งหมด 924 bp (307 กรดอะมิโน) และให้ชื่อว่า *bpsII* ปริมาณ GC content สูงตลอดสาย มี predicted pI = 5.8 และ estimated molecular weight = 33.5 KDa

จากนั้นนำ predicted amino acids มาศึกษา homology กับโปรตีนที่รู้ลำดับกรดอะมิโนแล้วใน EMBL database พบว่ามีความคล้ายคลึงกับ glyoxalaseII จาก *Arabidopsis thaliana* (32 % identity) [20] และ metallo β-lactamases (class B β-lactamases) subgroup B3 เช่น THIN-B[21], FEZ-1[22], LI[23] และ GOB-1[24] (30%, 27%, 26% และ 24% identity ตามลำดับ)

```
        10         20         30         40         50         60
ATGGGCTCCAGGAGAAACCTCGTGACAACAGCGTCCACGATGACGGTCGAAGGCTTTTTC
 M  G  S  R  R  N  L  V  T  T  A  S  T  M  T  V  E  G  F  F
        70         80         90        100        110        120
GACCCGGCGACATGCACGATCAGCTATTTGCTGTTCGATTCCGGCAGCGGCGAATGCGCG
 D  P  A  T  C  T  I  S  Y  L  L  F  D  S  G  S  G  E  C  A
       130        140        150        160        170        180
CTGATCGACAGCGTGCTCGACTACGACCCGAAATCCGGCCGCACGCGCACCGCGAGCGCG
 L  I  D  S  V  L  D  Y  D  P  K  S  G  R  T  R  T  A  S  A
       190        200        210        220        230        240
GACCAGTTGATCGCGCGCGTCGCGGCGCTCGGCGCGCGCGTGCGCTGGCTGCTCGAGACG
 D  Q  L  I  A  R  V  A  A  L  G  A  R  V  R  W  L  L  E  T
       250        260        270        280        290        300
CACGTGCACGCCGACCATCTGTCGGCCGCGCCGTACCTGAAAACGCGGGTCGGCGGCGAG
 H  V  H  A  D  H  L  S  A  A  P  Y  L  K  T  R  V  G  G  E
       310        320        330        340        350        360
ATCGCGATCGGCCGCCACGTGACGCGCGTGCAGGACGTGTTCGGCAAGCTGTTCAACGCA
 I  A  I  G  R  H  V  T  R  V  Q  D  V  F  G  K  L  F  N  A
       370        380        390        400        410        420
GGCCCCGCGTTCGCGCACGACGGCAGCCAGTTCGACCGCCTGCTCGACGACGGCGACACG
 G  P  A  F  A  H  D  G  S  Q  F  D  R  L  L  D  D  G  D  T
       430        440        450        460        470        480
CTCGCGCTCGGCGCGCTCTCGATCCGCGCGATGCATACGCCGGGCCACACGCCGGCGTGC
 L  A  L  G  A  L  S  I  R  A  M  H  T  P  G  H  T  P  A  C
       490        500        510        520        530        540
ATGACCTACGTCGTCACCGAAGCGCATGCGGCGCACGACGCGCGCGACGCGGGCGCGTTC
 M  T  Y  V  V  T  E  A  H  A  A  H  D  A  R  D  A  G  A  F
       550        560        570        580        590        600
GTCGGCGATACGCTGTTCATGCCCGACTACGGCACCGCGCGCTGCGACTTCCCCGGCGGC
 V  G  D  T  L  F  M  P  D  Y  G  T  A  R  C  D  F  P  G  G
       610        620        630        640        650        660
GACGCGCGCTCGCTGTACCGGTCGATCCGCAAGGTGCTGAGCCTGCCGCCCGCGACGCGC
 D  A  R  S  L  Y  R  S  I  R  K  V  L  S  L  P  P  A  T  R
       670        680        690        700        710        720
CTGTACATGTGCCACGACTATCAGCCGAACGGCCGCGCGATCCAGTACGCGAGCACCGTC
 L  Y  M  C  H  D  Y  Q  P  N  G  R  A  I  Q  Y  A  S  T  V
       730        740        750        760        770        780
GCCGACGAGTTGCGCGAGAACGTGCACATCCGCGAAGGCGTCACCGAGGACGATTTCGTC
 A  D  E  L  R  E  N  V  H  I  R  E  G  V  T  E  D  D  F  V
       790        800        810        820        830        840
GCGATGCGCACCGCGCGCGACGCGACGCTCGACATGCCGGTGCTGATGCTGCCCTCGGTG
 A  M  R  T  A  R  D  A  T  L  D  M  P  V  L  M  L  P  S  V
       850        860        870        880        890        900
CAGGTCAACATGCGCGCGGGCCGCCTGCCCGAGCCCGAGGACAACGGCGTGCGCTACCTG
 Q  V  N  M  R  A  G  R  L  P  E  P  E  D  N  G  V  R  Y  L
       910        920        930
AAGATCCCGCTCGACGCGATCTGA
 K  I  P  L  D  A  I  *
```

รูป 13. Nucleotide and deduced amino acid sequences of class B-like β-lactamase from *B. pseudomallei* strain 1902a.

14. การโคลน class B-like β-lactamase จาก *B. pseudomallei* โดยวิธี TOPO® Cloning

เนื่องจาก PCR product ที่ได้จากเชื้อ *B. pseu*domallei สายพันธุ์ 1902a และ E15 มีขนาดใกล้เคียงกับ class B-like β-lactamase gene และเป็น ผลิตผลหลักที่ได้โดยที่ไม่มี DNA อื่นปน ขั้นตอนต่อไปคือการนำชิ้นส่วน DNA นี้ไปต่อกับเวคเตอร์ pCR® T7/CT-TOPO® และ transform เข้าไปใน *E. coli* TOP10F'

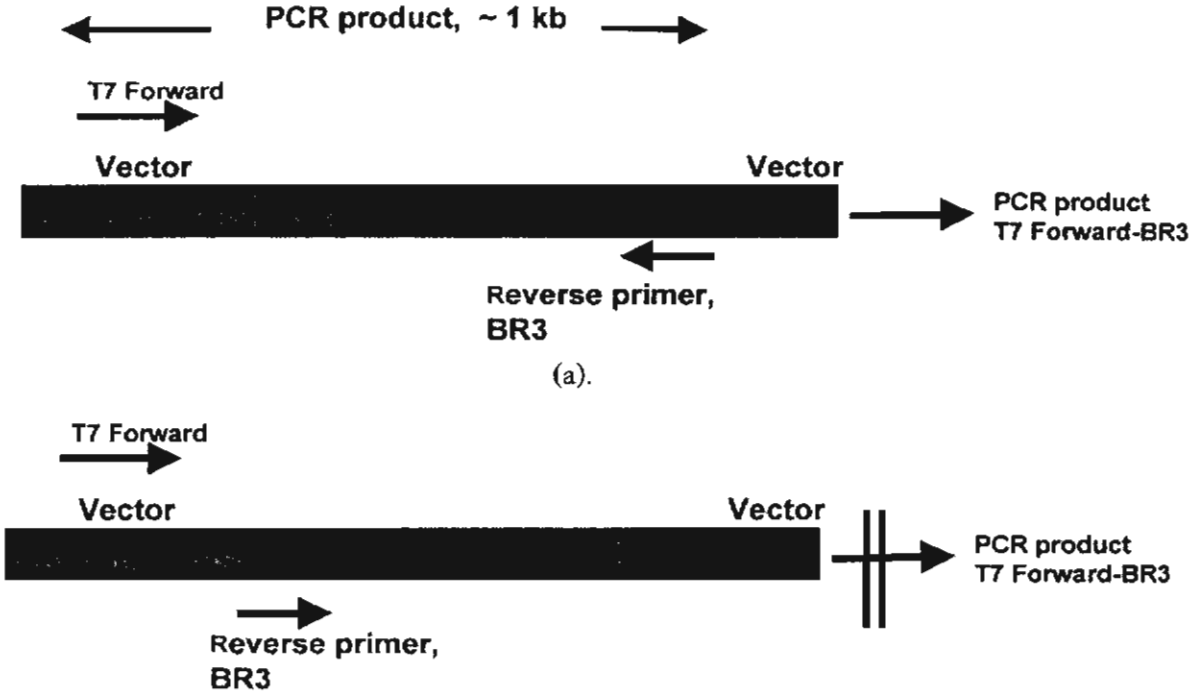
เมื่อได้โคโลนีของ *E. coli* TOP10F' ที่มี recombinant plasmid อยู่แล้วก็ต้องมีการการตรวจสอบทิศทางของ insert ที่เข้าไปรวมกับเวคเตอร์เช่นเดียวกับการโคลน class D β-lactamase gene ดังนั้นจึงต้องมีการตรวจสอบโคลนที่ได้จากว่ามีการรวมตัวกันของ PCR product และเวคเตอร์อย่างถูกทิศทางหรือไม่ ซึ่งสามารถทำได้โดยใช้วิธี PCR (ข้อ 8) ในกรณีนี้ template ที่ใช้คือ recombinant plasmid ที่สกัดออกมาจาก *E. coli* TOP10F' ส่วน primers ที่ใช้คือ

forward primer: ใช้ primer ที่จับกับเบสในส่วนของเวคเตอร์ ในที่นี้คือ T7 forward ซึ่งมีลำดับเบสดังนี้ 5'-TAATACGACTCACTATAGGG-3'

reverse primer: ใช้ primer ที่จับกับเบสในส่วนปลายของ PCR product ในที่นี้คือ BR3

โดยอาศัยหลักการที่ว่าเมื่อใช้ T7 forward และ BR3 เป็น primer จะให้ PCR product ซึ่งมีขนาดประมาณ 1 kb ถ้า insert เข้ารวมกับเวคเตอร์อย่างถูกทิศทาง (รูป 14a) แต่จะไม่เกิด PCR product ถ้า insert เข้ารวมกับเวคเตอร์ผิดทิศทาง (รูป 14b)

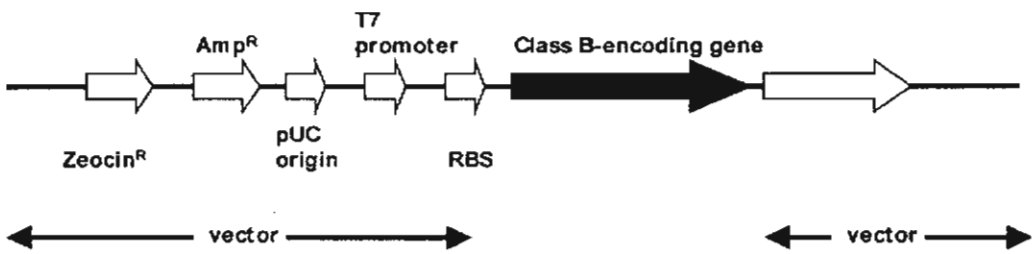
ซึ่งก็จะได้โคลน *E. coli* TOP10F' ที่มี class B β-lactamase-like gene จากเชื้อ *B. pseu*domallei สายพันธุ์ 1902a และ E15 ต่ออยู่กับเวคเตอร์ในลักษณะ correct orientation โดยจะมีโครงสร้างดังรูป 15



รูป 14 แสดงการตรวจสอบทิศทางการเข้ารวมกันระหว่าง PCR product กับเวคเตอร์ pCR® T7/CT-TOPO® ในปฏิกิริยาลูกโซ่โพลิเมอร์เรสโดยใช้ Forward primer คือ T7 forward และ Reverse primer คือ BR3

(a). การรวมตัวที่ถูกทิศทางจะเกิด PCR product

(b). การรวมตัวที่ผิดทิศทางโดยจะไม่เกิด PCR product



รูป 15 แสดงโครงสร้างของ pCR® T7/CT-TOPO® -*bpsII*

ZeocinR=Zeocin-resistant gene, AmpR=Ampicillin-resistant gene, RBS= Ribosome Binding Site

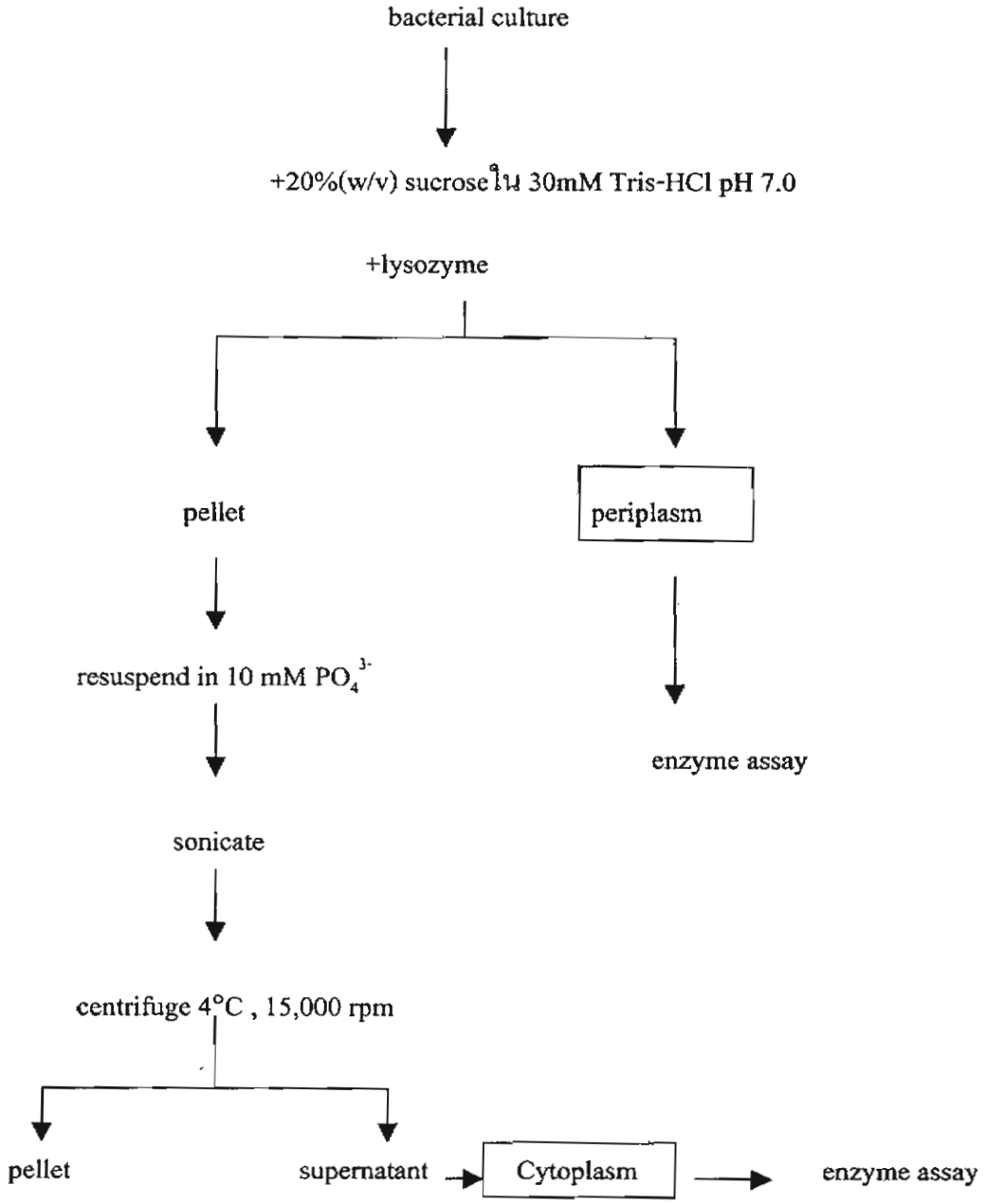
15. การหา β-lactam susceptibilities และ β-lactamase activity ของ *E. coli* ที่มี class B β-lactamase-like gene

เช่นเดียวกันกับการหา β-lactamase activity ของ *E. coli* ที่มี β-lactamase- class D gene (ข้อ 9) โดยในขั้นแรกก็จะทำการสกัด recombinant plasmid แล้ว transform เข้าไปใน *E. coli* BL21(DE3) pLysS (Invitrogen) ก่อนเพื่อที่จะได้มีการแสดงออกของยีนมากขึ้น จากนั้นนำมาหาการหา β-lactam susceptibilities (MIC) ด้วยวิธี Agar dilution method และหา β-lactamase activity เปรียบเทียบกับ *E. coli* ที่ไม่มียีนใดอยู่

โดยทั่วไปแล้วเอนไซม์ β-lactamase ในแบคทีเรียแกรมลบจัดเป็น periplasmic enzyme ซึ่งในสายโพลีเปปไทด์นั้นจะต้องมีโครงสร้างที่เรียกว่า signal sequence อยู่ที่บริเวณ N-terminal (รูป 16) โดย signal sequence นี้จะประกอบไปด้วย hydrophobic amino acid หลายๆตัวมาต่อกันประมาณ 22-24 ตัว ซึ่งโครงสร้างส่วนนี้จะทำให้เอนไซม์ β-lactamase สามารถแทรกตัวผ่านชั้นของฟอสโฟลิปิดที่เซลเมมเบรนออกไปสู่บริเวณ periplasm ได้ แต่เมื่อได้ทำการวิเคราะห์ลำดับของกรดอะมิโนของ class B β-lactamase-like จากเชื้อ *B. pseudomallei* สายพันธุ์ 1902a อย่างละเอียดโดยใช้โปรแกรม SignalP (http://www.cbs.dtu.dk/services/SignalP/) แล้วพบว่าไม่มี signal sequence ดังนั้นเอนไซม์นี้จึงอาจจัดว่าเป็น cytoplasmic enzyme

รูป 16 แสดงโครงสร้างของเอนไซม์ β-lactamase

ดังนั้นจึงทำการตรวจสอบ class B β-lactamase carbapenemase activity ในชั้นของ periplasm แยกจากไซโตพลาสซึมโดยทำการแยก periplasm ตามวิธีการในรูป 17 จากนั้นนำสารสกัดจาก periplasm และไซโตพลาสซึมมาตรวจหา activity ของเอนไซม์เปรียบเทียบกัน ผลการทดลองแสดงในตารางที่ 6



รูป 17 แสดงไดอะแกรมการสกัดเอนไซม์ β-lactamase ในชั้นของ periplasm และไซโตพลาสซึม

ตาราง 6 แสดง Specific Activity ของ *E. coli* BL21 (DE3) pLysS (*bpsII*-1902a) หรือ (*bpsII*-E15) ในชั้นของ periplasm และไซโตพลาสซึม

strains	Meropenem		Imipenem		Ampicillin	
	Cyto plasm	Peri plasm	Cyto plasm	Peri plasm	Cyto plasm	Peri plasm
BL21(DE3)pLysS	2.4 ± 0	0	0.8 ± 0.4	0	15.9±3.5	0
BL21(DE3)pLysS(*bpsII*-1902a)	9.5 ± 1.4	0	2.6 ± 0	0	ND	5652 ± 247
BL21(DE3)pLysS (*bpsII*-E15)	8.8 ± 0.7	0	3.1 ± 0.1	0	ND	904 ± 255

หมายเหตุ ND= No Detectable activity

* มีหน่วยเป็น μ mole substrate hydrolysed min^{-1} mg $protein^{-1}$

bpsII-1902a=classB β-lactamase-like gene จาก *B. pseudomallei* สายพันธุ์ 1902a

bpsII-E15= classB β-lactamase-like gene จาก *B. pseudomallei* สายพันธุ์ E15

จากตาราง 6 พบว่าสารสกัดไซโตพลาสซึมจาก *E. coli* BL21(DE3) pLysS (*bpsII*-1902a) หรือ (*bpsII*-E15) สามารถย่อยสลาย carbapenem ได้เมื่อเปรียบเทียบกับกลุ่มควบคุมคือ เชื้อ *E. coli* BL21(DE3) pLysS ที่ไม่มี recombinant plasmid อยู่ จากผลการทดลองสามารถสรุปได้ดังนี้

1. activity ต่อ imipenem และ meropenem ของ *E. coli* BL21(DE3) pLysS (*bpsII*-1902a) และ (*bpsII*-E15) จะอยู่ในชั้นของไซโตพลาสซึม แต่จะไม่พบใน periplasm
2. activity ต่อ ampicillin ของ *E. coli* BL21(DE3) pLysS (*bpsII*-1902a) และ (*bpsII*-E15) นั้นพบมากในชั้นของ periplasm ซึ่งเป็นผลมาจาก ampicillin-hydrolysing enzyme จากเวคเตอร์ ในส่วนของไซโตพลาสซึมก็พบ activity ต่อ ampicillin บ้างเช่นกัน (แต่จะน้อยกว่าใน periplasm มาก)

ซึ่งทั้ง carbapenemase และ ampicillinase activity นี้น่าจะเป็นผลมาจากยีน *bpsII* จาก recombinant plasmid

3. สารสกัดจาก periplasm ไม่มี activity ต่อ carbapenem

เนื่องจาก เอนไซม์ carbapenemase ในแบคทีเรียกรัมลบนั้นจะอยู่ในส่วนของ periplasm ซึ่งจากการผลการทดลองนี้ชี้ให้เห็นว่าตำแหน่งของ BpsII นั้นน่าจะอยู่ในชั้นของไซโตพลาสซึม แสดงให้เห็นว่า BpsII นั้นอาจไม่ใช่ true carbapenemase แต่อาจมีหน้าที่อื่นเช่นทำหน้าที่เป็น glyoxalase II ซึ่งก็จะต้องมีการศึกษาต่อไป

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Output ที่ได้จากโครงการ

- นำเสนอในการประชุม World Melioidosis Congress ณ เมือง Perth ประเทศออสเตรเลีย เมื่อวันที่ 26-29 กันยายน 2544 (ภาคผนวก 1)
- นำเสนอในการประชุมวิชาการของสำนักงานกองทุนสนับสนุนการวิจัย ในเดือน ตุลาคม 2544 ณ โรงแรมเฟลิกซ์ริเวอร์แคว จ. กาญจนบุรี โดยได้รับรางวัลการเสนอผลงานวิจัยดีเด่น (ภาคผนวก 2)
- นำเสนอในการประชุมวิชาการของมหาวิทยาลัยนเรศวรเมื่อเดือนกุมภาพันธ์ 2545 (ภาคผนวก 3)
- ตีพิมพ์ในวารสารระดับนานาชาติ Journal of Antimicrobial Chemotherapy (ภาคผนวก 4)

ภาคผนวก

World Melioidosis Congress

incorporating the inaugural

Emerging Infectious Diseases of the Indian Ocean Rim (EIDIOR) Workshop

26th - 29th September, 2001
Burswood International Resort Convention Centre
Perth, Western Australia

Delegate Handbook
and Abstracts

Abstract 87

CEFTAZIDIME- AND IMIPENEM- HYDROLYZING BETA-LACTAMASE FROM *BURKHOLDERIA PSEUDOMALLEI*

Niumsup P[1] *& Wuthiekanun V*[2]

[1]Department of Microbiology, Faculty of Medicine, Naresuan University, Phitsanuloke 65000, Thailand

[2]Wellcome Trust Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

The antibiotic of choice for the treatment of melioidosis is ceftazidime. Ceftazidime-resistant *Burkholderia pseudomallei* has been identified and ß-lactamases implicated in resistance. In this study, 20 strains of *B. pseudomallei* (12 clinical and 8 environmental strains) were examined for their ability to yield mutants derepressed for ß-lactamase expression. Ceftazidime-resistant mutants were readily selected, by plating organisms on ceftazidime-containing media, at high frequencies and displayed a 4-8 fold increase in MICs against ceftazidime, as determined by the agar dilution method. ß-Lactamase activities in both parent and mutant *B. pseudomallei* strains were determined by a spectrophotometric method. All strains tested possess ß-lactamase activity against ampicillin. 12 mutants (7 clinical and 5 environmental strains) had approximately 2-10 fold higher ceftazidimase activity compared with their parent strains. Furthermore, 12 of these mutants (60%) demonstrated a significant increase in imipenemase activity, usually indicative of a metallo ß-lactamase. ß-Lactamase induction in *B. pseudomallei* was also investigated using ceftazidime as an inducer. Preliminary results show that moderate level ß-lactamase expression (against ampicillin, ceftazidime and imipenem) could be induced with sub-MIC levels of ceftazidime. The fact that a single inducer can cause an increase in expression of multiple ß-lactamase activities, suggests that the expression of these enzymes are linked. More strains are currently being investigated for ß-lactamase induction to obtain a further understanding of how these clinically important enzymes are controlled.

ภาคผนวก 2

กำหนดการและบทคัดย่อ

การประชุมเพื่อเสนอผลงานวิจัย โครงการทุนวิจัยหลังปริญญาเอก ครั้งที่ ๒

ISBN 974-8196-98-4

ณ โรงแรมเฟลิกซ์ จ.กาญจนบุรี

ระหว่างวันที่ 12-14 ตุลาคม 2544

สำนักงานกองทุนสนับสนุนการวิจัย

The Thailand Research Fund

TITLE: CEFTAZIDIME- AND IMIPENEM- HYDROLYZING BETA-LACTAMASE FROM *Burkholderia pseudomallei*

AUTHOR (S): Pannika Niumsup[1] and Vannaporn Wuthiekanun[2]

ADDRESS: [1]Department of Microbiology, Faculty of Medicine, Naresuan University, Phitsanuloke 65000, Thailand. [2]Wellcome Trust Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Objective: To investigate the β-lactam resistance in *B. pseudomallei*.

Methods: Ceftazidime-resistant *B. pseudomallei* strains were selected, by plating organisms on ceftazidime-containing agar. MICs and β-lactamase activities of both parent and mutant strains were examined by agar dilution method, according to NCCLS guidelines, and spectrophotometric method, respectively.

Results: 20 strains of *B. pseudomallei* (12 clinical and 8 environmental strains) were examined for their ability to yield mutants derepressed for β-lactamase expression. Ceftazidime-resistant mutants were readily selected at high frequencies and displayed a 4-8 fold increase in MICs against ceftazidime. All strains tested possess activities against ampicillin. 12 mutants (7 clinical and 5 environmental strains) had approximately 2-10 fold higher ceftazidimase activity compared with their parent strains. Furthermore, 12 of these mutants (60%) demonstrated a significant increase in imipenemase activity, usually indicative of a metallo β-lactamase. β-Lactamase induction in *B. pseudomallei* was also investigated using ceftazidime as an inducer. Preliminary results show that moderate level β-lactamase expression (against ampicillin, ceftazidime and imipenem) could be induced with sub-MIC levels of ceftazidime.

Conclusion: The presence of β-lactamases in *B. pseudomallei* strains suggested the involvement of these enzymes in β-lactam resistance. The fact that a single inducer can cause an increase in expression of multiple β-lactamase activities, suggest that the expression of these enzymes may be co-ordinately controlled.

Selected references:

1. Livermore D.M., Chau P.Y., Wong C.I. and Leung Y.K. (1987). β-Lactamases of *Pseudomonas pseudomallei* and its contribution to antibiotic resistance. J Antimicrob Chemother 20,313-321.

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Keywords: *Burkholderia pseudomallei*, β-lactamases, ceftazidime

บันทึกข้อความ

ส่วนราชการ สำนักงานอธิการบดี กองบริการการศึกษา งานวิจัย โทร. ๑๑๔๖
ที่ ทม ๑๙๐๑.๐๓(๓)/ ๒๙๐๗ **วันที่** ๒๑ มกราคม ๒๕๔๕
เรื่อง ขอเรียนเชิญนำเสนอโครงการวิจัยที่ได้รับรางวัล

เรียน ดร.พรรณิกา เนียมทรัพย์

ด้วยมหาวิทยาลัยนเรศวร จะจัดแสดงนิทรรศการโครงงานของนิสิตมหาวิทยาลัยนเรศวร ประจำปี ๒๕๔๕ และการสัมมนาทางวิชาการเรื่อง "ทิศทางการวิจัยของมหาวิทยาลัยนเรศวรในปัจจุบันและอนาคต" ขึ้นในระหว่างวันที่ ๑๘–๒๐ กุมภาพันธ์ ๒๕๔๕ ณ ศูนย์บริการเทคโนโลยีสารสนเทศและการสื่อสาร รายละเอียดตามโครงการแนบท้าย

เพื่อเป็นการเผยแพร่ผลงานวิจัยของท่านและสร้างแรงจูงใจในการทำงานวิจัยให้กับนิสิตและอาจารย์ ในการนี้มหาวิทยาลัยใคร่ขอเรียนเชิญท่านนำเสนอโครงการวิจัยที่ได้รับรางวัล ประมาณ ๑๕ นาที ในวันที่ ๒๐ กุมภาพันธ์ ๒๕๔๕ ระหว่างเวลา ๐๙.๐๐–๑๐.๐๐ น. ณ ศูนย์บริการเทคโนโลยีสารสนเทศและการสื่อสาร ห้อง Main Conference ตามกำหนดการที่แนบมาพร้อมนี้

จึงเรียนมาเพื่อโปรดพิจารณา และแจ้งผลตอบรับมายังงานวิจัย ภายในวันที่ ๒๕ มกราคม ๒๕๔๕ หวังว่าคงได้รับความอนุเคราะห์จากท่านด้วยดี จึงขอขอบคุณมา ณ โอกาสนี้

(ศาสตราจารย์ ดร.บรรพต สุวรรณประเสริฐ)
รองอธิการบดีฝ่ายวิจัย

กำหนดการ

การแสดงนิทรรศการโครงงานของนิสิตมหาวิทยาลัยนเรศวร

และ

การสัมมนาทิศทางการวิจัยของมหาวิทยาลัยนเรศวรในปัจจุบันและอนาคต

ระหว่างวันที่ 18-20 กุมภาพันธ์ 2545

ณ ศูนย์บริการเทคโนโลยีสารสนเทศและการสื่อสาร ห้อง Main Conference

****

<u>วันจันทร์ที่ 18 กุมภาพันธ์ 2545</u>

08.30-09.00 น. - ลงทะเบียน

09.00-09.30 น. - เปิดการแสดงนิทรรศการ

- รองอธิการบดีฝ่ายวิจัยกล่าวรายงาน / อธิการบดีกล่าวเปิดงาน
- การแสดงออกซึ่งวัฒนธรรมไทย

10.00-16.00 น. - แขกผู้มีเกียรติร่วมชมนิทรรศการ

13.00-16.00 น. - คณะกรรมการให้คะแนนโครงงาน/สัมภาษณ์โครงงาน

<u>วันอังคารที่ 19 กุมภาพันธ์ 2545</u>

09.00-09.15 น. - ลงทะเบียน

09.15-09.30 น. - กล่าวต้อนรับและพิธีเปิด โดย อธิการบดีมหาวิทยาลัยนเรศวร

09.30-10.30 น. - บรรยายหัวข้อ "ทิศทาง ส่งเสริม สนับสนุน การวิจัยของมหาวิทยาลัยนเรศวร" โดย **รศ.ดร.มณฑล สงวนเสริมศรี อธิการบดี**

10.30-10.45 น. - พักรับประทานอาหารว่าง

10.45-12.00 น. - บรรยายหัวข้อ "แนวโน้ม จุดเน้น และการเสนอโครงการวิจัย เพื่อขอรับทุนสนับสนุนจาก สำนักงานกองทุนสนับสนุนการวิจัย
โดย รองผู้อำนวยการสำนักงานกองทุนสนับสนุนการวิจัย
(ผู้ช่วยศาสตราจารย์วุฒิพงศ์ เตชะดำรงสิน)

12.00-13.00 น. - **พักรับประทานอาหารกลางวัน**

13.00-14.45 น. - บรรยายหัวข้อ "แนวโน้ม จุดเน้น และการเสนอโครงการวิจัย เพื่อขอรับทุนสนับสนุนจาก สำนักงานคณะกรรมการวิจัยแห่งชาติ
โดย รองเลขาธิการคณะกรรมการวิจัยแห่งชาติ
(นายชอบวิทย์ ลับไพรี)

14.45-15.00 น. - พักรับประทานอาหารว่าง

วันอังคารที่ 19 กุมภาพันธ์ 2545 (ต่อ)

15.00-16.00 น. - สรุปอภิปรายและตอบประเด็นซักถามโดย
ผู้อำนวยการสำนักงานกองทุนสนับสนุนการวิจัย
เลขาธิการคณะกรรมการวิจัยแห่งชาติ
รองอธิการบดีฝ่ายวิจัย

16.00-16.30 น. - พิธีปิดและมอบของที่ระลึก

วันพุธที่ 20 กุมภาพันธ์ 2545

08.30-09.00 น. - ลงทะเบียน

09.00-10.00 น. - การนำเสนอโครงการวิจัยที่ได้รับรางวัล (คนละ 15 นาที)

❋ เรื่อง "การใช้จุลินทรีย์ร่วมการกระตุ้นพืชในการสร้างภูมิคุ้มกัน"
โดย ผศ.ดร.กัลชลี เจดิยานนท์
ได้ทุนสนับสนุนการวิจัยหลังปริญญาเอกจาก สกว. และได้รับรางวัล
การเสนอผลงานวิจัยดีเด่น ในปี 2544

❋ เรื่อง "ผลของเอมไซม์ β-Lactamase ต่อความสามารถในการต้านยา β-Lactam"
โดย ดร.พรรณิกา เนียมทรัพย์
ได้ทุนสนับสนุนการวิจัยหลังปริญญาเอกจาก สกว. และได้รับรางวัล
การเสนอผลงานวิจัยดีเด่น ในปี 2544

❋ เรื่อง "ริงที่มอดูลเอกฐานเป็นวีคลี่อินเจคทีฟ"
โดย รศ.ดร.สมยศ พลับเที่ยง
ได้รับรางวัลชมเชยจากสำนักงานคณะกรรมการวิจัยแห่งชาติ
ประเภทวิทยานิพนธ์ประกอบการศึกษาระดับปริญญาเอก ในปี 2544

10.00-11.00 น. - ประกาศผลและมอบรางวัลโครงงานของนิสิต
โดยอธิการบดีมหาวิทยาลัยนเรศวร (รศ.ดร.มณฑล สงวนเสริมศรี)

11.00-11.20 น. - พิธีปิดงานแสดงนิทรรศการโครงงานของนิสิต
โดยรองอธิการบดีฝ่ายวิจัย (ศ.ดร.บรรพต สุวรรณประเสริฐ)

****

Characterization of beta-lactamases in *B. pseudomallei*

Pannika Niumsup* and Vanaporn Wuthiekanun**

*Department of Microbiology and Parasitology, Faculty of Medical Sciences, Naresuan University, Phitsanuloke, 65000.

**Wellcome Trust Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok.

Burkholderia pseudomallei

-first isolated in 1912, motile G- bacillus

-formerly known as *Pseudomonas aeruginosa*

-environmental saprophyte

-abscess-forming infections

(Melioidosis)

Wellcome Trust Unit

Melioidosis

-A rainy season disease (June-November)

-Affects all ages (40-59)

-Male more than female (3:2)

-Affects predominantly rice farmers and their families

-Risk factors: diabetic, chronic renal failure, smoking, alcoholism

Melioidosis

-Endemic area: N/E Thailand& NT. Australia

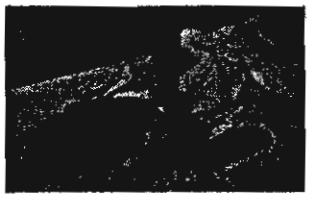
-Systemic infection: abscesses in lungs, spleen, liver, kidney, muscle etc.

-Septicaemia

-Mortality rate 40%

-beta-lactams: Ceftazidime & imipenem

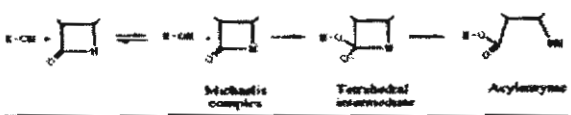
Melioidosis



UBON RATCHATANI
1986-1997
1504 patients(233 children)
(Wellcome Trust Unit)

- Mortality 51%
- Male 58%
- Known underlying disease 55%
- Septicaemic 62%

Beta-lactamases

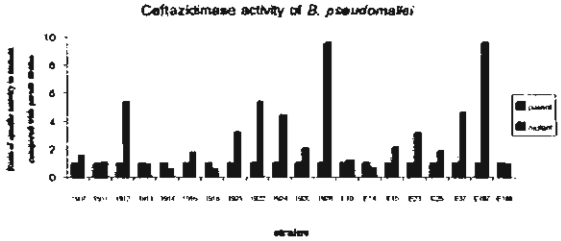


Results

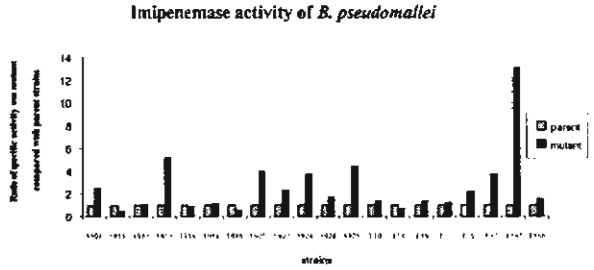
Table 1. Beta-lactam MICs of *B. pseudomallei*

Strains	ceftazidime (ug/ml)	Imipenem (ug/ml)
Parent	1	1
Mutants	4-8	1-4

Ceftazidimase activity in *B. pseudomallei*



Imipenemase Activities



Classification

Class A, C, D : serine beta-lactamases

Class B: metallo beta-lactamses

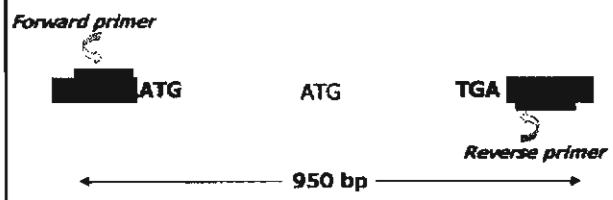
Beta-lactamase gene in *B. pseudomallei*

* class A beta-lactamase gene

* class D beta-lactamase gene

(Sanger Institute, Cambridge, UK)

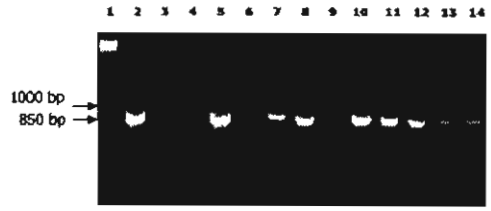
Amplification of the classD beta-lactamase gene



Cloning of the classD beta-lactamase gene

TOPO Cloning

Prevalence of classD beta-lactamase gene



Expression of classD beta-lactamase gene

B. pseudomallei **Parent & Mutant**

RNA isolation

↓ Reverse transcriptase

cDNA synthesis

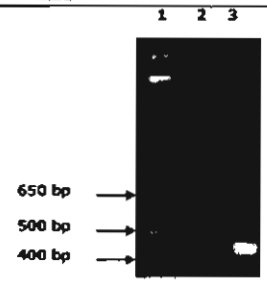
↓

PCR

↓

Agarose gel electrophoresis

Expression of classD beta-lactamase gene



RT-PCR

Conclusions

1. Beta-lactam-resistant *B. pseudomallei* possess activity against ceftazidime, imipenem

2. Class D beta-lactamase gene is involved in the beta-lactam resistant *B. pseudomallei*

Acknowledgements

1. Thailand Research Fund

2. British Society for Antimicrobial Chemotherapy (BSAC)

Acknowledgements

- Wellcome Trust Unit
  - Prof. Nicholas J. White
  - Vanaporn Wuthiekanun
- Bristol,UK
  - Dr. Timothy R. Walsh
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Perth, Australia
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Cloning of the class D β-lactamase gene from *Burkholderia pseudomallei* and studies on its expression in ceftazidime-susceptible and -resistant strains

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Ceftazidime is the antibiotic of choice for the treatment of melioidosis. Ceftazidime-resistant *Burkholderia pseudomallei* have been identified and β-lactamase production implicated in resistance. In this study, 25 strains of *B. pseudomallei* (15 clinical and 10 environmental strains) were examined for their ability to yield mutants that overexpress β-lactamase. Ceftazidime-resistant mutants were selected readily at high frequency and displayed four- to eight-fold increases in the MICs of ceftazidime. β-Lactamase activities in both parent and mutant *B. pseudomallei* strains were examined by a spectrophotometric method. Twelve mutants (48%) showed approximately two- to 31-fold higher ceftazidimase activity compared with their parent strains and 10 (40%) demonstrated more than two-fold increases in imipenemase activity. A class D β-lactamase gene from *B. pseudomallei* was cloned and sequenced. The encoded enzyme is an oxacillinase and is homologous to oxacillinases from *Ralstonia pickettii* and members of the genus *Aeromonas*. Reverse transcriptase PCR showed that transcription of the class D β-lactamase gene is increased in ceftazidime-resistant mutants.

Introduction

Melioidosis, a fatal disease caused by *Burkholderia pseudomallei*, is endemic in Thailand, especially in the north-east. Patients with systemic *B. pseudomallei* infection require prompt antimicrobial therapy, as the mortality rate is exceptionally high.[1] Currently, the antibiotic of choice for the treatment of acute melioidosis is ceftazidime.[2] Resistance to ceftazidime was recognized soon after this antibiotic became the treatment of choice for severe melioidosis. Resistance was shown to be the result of β-lactamase production.[3,4] The carbapenems were therefore evaluated as an alternative treatment, although their use is still limited.[5] Furthermore, in some other Gram-negative pathogens such as *Enterobacter cloacae*[6] and *Acinetobacter baumannii*,[7,8] carbapenem resistance has emerged, particularly because of the production of specific β-lactamases.

To date, there is relatively little information on β-lactamase expression in *B. pseudomallei*. In 1987, Livermore *et al.*[9] reported that nine strains of *B. pseudomallei* possess a weakly inducible cephalosporinase that is active against carbenicillin, cefotaxime and cefuroxime. Later, Dance *et al.*[3,4] noted the presence of a clavulanic acid-susceptible ceftazidimase. Godfrey *et al.*[10] reported that β-lactam resistance in *B. pseudomallei* resulted from overexpression of chromosomal β-lactamases, and this has also been shown to result in high-level resistance to β-lactams in *Burkholderia cepacia*.[11] In contrast, 194 strains of *B. pseudomallei* were examined for β-lactamase expression by Sookpranee *et al.*,[12] who concluded that the β-lactamases found were non-inducible.

Carbapenemases have recently become more prominent among β-lactam-hydrolysing enzymes, and have also been described in carbapenem-resistant *B. cepacia*.[13] This organism produces an inducible metallo-β-lactamase, designated PCM-1, that hydrolyses imipenem, meropenem and, to a lesser extent, ceftazidime. Therefore, it is not unreasonable to speculate that carbapenem-hydrolysing enzymes may also be present in *B. pseudomallei*. However, sequencing of the genome of *B. pseudomallei* strain K96243 has revealed the

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presence of class A, C and D β-lactamase genes, but not a class B (metallo-)β-lactamase gene (Sanger Institute, Cambridge, UK). The phenotypic significance of the β-lactamase genes located has not yet been elucidated.

When bacteria carrying a β-lactamase gene(s) are exposed to β-lactams, they come under strong selective pressure to develop resistance. Many Gram-negative bacteria, including *B. cepacia*, have been reported to produce, at high frequencies, mutants that overexpress β-lactamase.[11] In this study, environmental and clinical strains of *B. pseudomallei* were examined for their abilities to yield mutants that overexpress β-lactamase expression. The frequency of mutation to ceftazidime resistance, and the β-lactam resistance profiles and levels of β-lactamase production of the resultant mutants, were investigated. The genes encoding class D β-lactamases were cloned and sequenced from two *B. pseudomallei* strains. Expression of this gene in one ceftazidime-resistant mutant compared with its parent was also measured.

Materials and methods

Bacterial strains

The clinical *B. pseudomallei* strains (1901a–1928a) used in this study were isolated from patients in Ubon Ratchatani, north-east Thailand. Environmental strains (E10–E188) were isolated from rice fields in the north-east of Thailand (Table 1).

Selection of ceftazidime-resistant mutants

Ceftazidime-resistant mutants were selected by plating an overnight culture of *B. pseudomallei* onto ceftazidime-containing Mueller–Hinton agar at 4 × MIC. The same culture was also diluted, plated on agar without antibiotic and incubated at 37°C to determine the viable cell count. The mutation frequency was calculated as the number of resistant colonies compared with the number of cells in the original culture.

Susceptibility testing

MICs were determined by a standard agar dilution method. Inocula were prepared by diluting an overnight culture of each strain. Using a multipoint inoculator, ~10^4 cells were spotted onto dried Mueller–Hinton agar containing serial dilutions of the appropriate antibiotic. Antibiotic concentrations ranging from 0.125 to 512 mg/L with doubling increments were used. After 18 h incubation at 37°C, the MIC was recorded as the lowest concentration of antibiotic that inhibited visible growth.

Preparation of crude cell extracts containing β-lactamase

The *B. pseudomallei* cultures were grown until mid-log phase. Then, toluene was added to the cultures in order to kill bacteria, as described by Livermore *et al.*[9] The cells were harvested and washed once with 10 mM phosphate buffer (pH 7.0). The cultures were then disrupted on ice by four cycles of 20 s sonications with 15 s rest intervals between cycles (Vibra Cell; Sonics & Materials Inc., Newtown, CT, USA), and centrifuged at 13 000 rpm at 4°C for 10 min (Beckman J2-MC; Beckman, CA, USA). The supernatant was used in β-lactamase assays.

Crude cell extracts from *Escherichia coli* carrying the oxacillinase gene were prepared by periplasmic extraction as described by Lindström *et al.*[14]

Table 1. Time and area of isolation of *B. pseudomallei* strains used in this study

Strains	Year of isolation	Area of isolation
1901a, 1902a, 1904a, 1907a, 1911a, 1912a, 1914b, 1916a, 1918a, 1921a, 1922a, 1923a, 1924a, 1926a, 1928a	1998	north-east Thailand
E10, E14, E15, E19, E23, E25, E26, E37, E187, E188	1990	north-east Thailand
60.66	1960	Australia
59.6	1959	–
55.135, 56.91	1955	Vietnam
53.238, 52.239	1952	Vietnam
52.237	1950	Vietnam
A202, A203	1947	Vietnam

–, no information available.

β-Lactamase assays

β-Lactamase activities were measured by monitoring hydrolysis of β-lactams by spectrophotometric assay (Spectronic Genesys™ 5; Milton Roy Company, Rochester, NY, USA) at a wavelength of optimal absorbance for the β-lactam ring of each drug.[9,15] Antibiotic solutions were prepared freshly in 10 mM phosphate buffer (pH 7.0). Ceftazidime, imipenem (Merck & Co., Inc., West Point, PA, USA), ampicillin and cefalothin (Sigma, St Louis, MO, USA) were assayed at concentrations of 100, 300, 500 and 100 μM, respectively. Prior to each assay, a background reading of each antibiotic was performed to ensure that any decrease in substrate was solely the result of adding the enzyme extracts. All assays were run in triplicate. The protein concentration was measured using a Bio-Rad protein assay based on the Lowry method (Bio-Rad, Hercules, CA, USA). Specific activities were calculated as nanomoles of β-lactam hydrolysed per minute per milligram of protein.

Amplification and cloning of class D β-lactamase genes from B. pseudomallei

Chromosomal DNA was isolated from *B. pseudomallei* parent strains 1902a and E15 using an Easy-DNA kit (Invitrogen, Groningen, The Netherlands), and was used directly as a template in PCR. The primers used were based on the genome sequence of *B. pseudomallei* K96243, which is available online (at http://www.sanger.ac.uk/Projects/B_pseudomallei/). PCR primers were purchased from Sigma-Genosys Ltd (Pampisford, UK). The class D gene primers were: (forward) 5′-ATGAGCCATTCCCCACTCTT-3′ and (reverse) 5′-TTTTGCCGTTCACGAAGAC-3′, which are 32 bp upstream from the start codon and 83 bp downstream from the stop codon, respectively. The predicted PCR product was ~900 bp. Reactions were performed with 50 μL of mixture containing 100 ng of template, 0.25 μM each oligonucleotide primer, 200 μM dNTPs and 1 U of SuperTaq (HT Biotechnology Ltd, Cambridge, UK) together with its reaction buffer. The conditions comprised one cycle at 94°C for 5 min, followed by 50 cycles at 94°C for 1 min, 55°C for 1.3 min and 72°C for 2.3 min, and a final elongation at 72°C for 10 min.

The amplified class D gene products generated by PCR were cloned into the pCR T7/CT-TOPO vector (Invitrogen), according to the manufacturer's instructions. The recombinant plasmids were transformed into chemically competent *E. coli* TOP10F′ cells by heat shock, as described by the supplier. Transformants were plated on nutrient agar containing ampicillin (100 mg/L) and zeocin (50 mg/L), and incubated at 37°C overnight. Ten colonies were picked randomly and recombinant plasmids were isolated using the Hybaid Recovery Plasmid Prep Kit (Hybaid, Teddington, UK). The orientation of the inserts was verified by PCR.

Measuring expression of class D β-lactamase genes in B. pseudomallei using reverse transcriptase–PCR (RT–PCR)

Total RNA from *B. pseudomallei*, both parent and mutant strains, was isolated by Hybaid RiboLyser Kit *BLUE* using a HYBAID RiboLyser Instrument. The RNA was quantified and cDNAs were synthesized using RevertAid H Minus M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania), according to the supplier's instructions. RNAs (5 μg) were mixed with specific primers, incubated at 70°C for 5 min and chilled on ice. Then, reaction buffer was added, followed by dNTPs to a final concentration of 1 mM. This reaction was incubated at 70°C for 5 min. Two hundred units of RevertAid H Minus M-MuLV Reverse Transcriptase were added and incubated at 42°C for 60 min. Finally, the reaction was terminated by heating at 70°C for 10 min and chilled on ice. The synthesized cDNA was used directly as template in RT–PCR without further purification. The oligonucleotide primers were designed within the gene: (forward) 5′-GCTGCTGGTGCAGGACGGCG-3′ and (reverse) 5′-CGTCATGTCGACGGCTGTG-3′. The predicted PCR product was 435 bp. Amplification reactions were performed as described earlier and the PCR conditions were as followed: one cycle at 94°C for 5 min, followed by 50 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 2 min, with a final elongation at 72°C for 10 min. The amplified products were analysed on 1% agarose gel electrophoresis.

Table 2. Frequencies of mutation and β-lactam susceptibilities of *B. pseudomallei* strains used in this study

Strains	Frequencies of mutation	Range of ceftazidime MIC (mg/L)		Range of imipenem MIC (mg/L)	
		parent	mutant	parent	mutant
Clinical (1901a–1928a)	10^{-6}–10^{-9}	1–2	4–8	1	1–4
Environmental (E10–E188)	10^{-9}–10^{-10}	1	4–8	1	1–2

Results

Isolation of ceftazidime-resistant B. pseudomallei mutants

Ceftazidime-resistant mutants of 25 *B. pseudomallei* strains (15 clinical and 10 environmental strains) were selected by plating overnight cultures of *B. pseudomallei* onto ceftazidime-containing agar. It should be noted that the 'ceftazidime-resistant mutant' in this article refers to strains having four- to eight-fold increases in the MIC of ceftazidime compared with the parent strain. The mutation frequencies determined are shown in Table 2.

Ceftazidime-resistant mutants from clinical isolates were selected at frequencies of 10^{-6}–10^{-9} (mostly 10^{-8}). For environmental isolates, the mutation frequencies (for selecting ceftazidime-resistant mutants) were slightly lower (10^{-9}–10^{-10}). The frequencies of mutant isolation suggested that point mutations leading to gene disruption were most likely to be responsible for the resistant phenotype, as seen in *B. cepacia*,[11] *Aeromonas* spp.[16] and *Stenotrophomonas maltophilia*.[17] One mutant of each strain was chosen randomly for further investigation.

β-Lactam susceptibilities and β-lactamase production of B. pseudomallei, parent and mutant strains

The MICs of ceftazidime and imipenem against 25 parent and ceftazidime-resistant *B. pseudomallei* mutants were determined. The ranges of MICs against all strains are shown in Table 2. The parent strains were susceptible to both ceftazid-

Table 3. β-Lactamase activities of *B. pseudomallei* parent strains and mutant progeny

	Specific activity against ceftazidime[a]			Specific activity against imipenem[a]		
Strains	parent	mutant	approximate fold increase[b]	parent	mutant	approximate fold increase[b]
1901a	6.1±1.5	170.9±11.9	28.0	32.4±1.8	137.8±40.2	4.3
1902a	10.7±1.6	339.1±110.9	31.7	7.3±2.4	383.6±33.2	52.5
1904a	8.9±1.6	4.5±1.1	–	9.0±1.8	4.6±0.7	–
1907a	13.0±2.5	20.6±1.5	–	16.6±6.9	42.4±11.0	2.6
1911a	19.9±0	26.6±0	–	15.8±5.3	8.5±1.7	–
1912a	4.9±2.6	26.4±6.2	5.4	19.1±3.0	20.3±4.9	–
1914b	19.3±5.0	11.8±5.2	–	10.8±1.7	9.5±0.6	–
1916a	3.5±1.2	6.4±1.8	–	5.1±2.3	5.8±2.5	–
1918a	6.0±1.9	3.2±0.4	–	7.4±0.9	4.5±0	–
1921a	24.6±0	107.7±25.4	3.2	27.8±6.3	110.1±11.2	4.0
1922a	5.8±3.3	31.3±6.4	5.4	2.2±0.4	4.9±1.6	2.3
1923a	6.0±1.9	3.8±1.1	–	8.5±2.8	4.8±1.2	–
1924a	5.6±2.1	25.0±0	4.5	15.4±1.7	57.6±15.6	3.7
1926a	3.3±1.3	16.5±7.0	5.0	3.4±1.2	5.9±0	–
1928a	1.2±0.7	11.3±3.5	9.6	6.1±1.1	26.8±8.1	4.4
E10	15.8±2.8	13.7±1.3	–	7.7±0.8	10.5±1.1	–
E14	6.4±1.4	4.3±1.0	–	9.1±0.8	6.8±0.6	–
E15	1.7±0	16.6±5.4	9.8	8.7±2.6	11.4±2.0	–
E19	4.4±0.9	2.2±1.3	–	6.6±0.9	5.2±0.5	–
E23	6.1±2.2	19.1±7.9	3.2	5.5±2.1	6.9±2.9	–
E25	4.4±1.5	8.3±1.0	–	4.3±1.6	9.6±2.1	2.2
E26	11.8±4.0	7.2±2.7	–	6.2±1.6	3.9±1.2	–
E37	2.1±0.4	9.7±3.6	4.6	3.9±0.9	14.4±0.9	3.7
E187	15.7±2.6	150.6±0	9.6	15.0±2.5	195.7±43.1	13.0
E188	15.3±2.8	12.4±1.9	–	10.8±1.2	10.8±1.3	–

The values given are the average number from three separate experiments. 1901a–1928a and E10–E188 are clinical and environmental strains, respectively.
[a]One unit: nanomoles of substrate hydrolysed per minute per milligram of protein.
[b]An increase in activity greater than two-fold is considered significant.

ime and imipenem. The mutants displayed up to eight- and four-fold increases in MICs of ceftazidime and imipenem, respectively.

To investigate whether the increase in MICs of ceftazidime and imipenem was the result of β-lactamase production, β-lactamase activities were determined, specifically against imipenem and ceftazidime. The imipenemase and ceftazidimase activities of 25 *B. pseudomallei* strains (both parent and mutant strains) are shown in Table 3. Twelve ceftazidime-resistant mutants (48%) (1901a, 1902a, 1912a, 1921a, 1922a, 1924a, 1926a, 1928a, E15, E23, E37, E187) showed two- to 31-fold increases in activities against ceftazidime. Increased imipenemase activity was found in 10 ceftazidime-resistant mutants (40%), which displayed two- to 52-fold higher activities compared with their parent strains (1901a, 1902a, 1907a, 1921a, 1922a, 1924a, 1928a, E25, E37, E187). Ceftazidime-resistant mutants of strains 1901a, 1902a, 1921a, 1922a, 1924a, 1928a, E37 and E187 (31% of all mutants) displayed a more than two-fold increase in activity against both imipenem and ceftazidime. Ceftazidime-resistant mutants of strains 1904a, 1911a, 1914b, 1916a, 1918a, 1923a, E10, E14, E19, E26 and E188, despite increases in MICs, displayed β-lactamase activities similar to their parents. Ceftazidime resistance in these mutants may have resulted from other mechanisms, e.g. alteration in outer membrane proteins or drug efflux.

Amplification, sequencing and cloning of class D β-lactamase genes from B. pseudomallei

The genome sequence of *B. pseudomallei* has revealed the presence of class A (contig 93), class C (contig 421) and class D (contig 14) β-lactamase genes. *B. pseudomallei* strains 1902a and E15 were randomly chosen as representatives of clinical and environmental strains, respectively, for cloning of the class D β-lactamase genes. Genomic DNAs were extracted and amplification of class D β-lactamase genes from *B. pseudomallei*, strains 1902a and E15, were performed. A single, discrete band of ~900 bp was obtained for each strain, which was consistent with the predicted PCR product for a class D β-lactamase gene.

Sequencing of the PCR product from strain 1902a revealed an open reading frame of 810 nucleotides, encoding a product of 269 amino acids. The nucleotide sequence of this open reading frame and its deduced amino acids are shown in Figure 1 (EMBL accession number AJ488302). The GC content is high throughout the open reading frame (65.9%), which corresponds to the overall GC content of the *B. pseudomallei* chromosome. This new class D β-lactamase, designated OXA-42, has a predicted pI of 9.4 and an estimated molecular weight of 29.5 kDa. Like other β-lactamases, the primary translation product has a strong hydrophobic N-terminus, typical of periplasmic proteins. The predicted cleavage site is likely to be between alanine and lysine at position 23–24. Within the enzyme, the conserved Ser-Thr-Phe-Lys active site was found at position 53–56. Four other conserved regions for class D β-lactamases were also identified (Figure 1): Tyr-Gly-Asn (position 130–132); Trp-Xaa-Gly-Xaa-Xaa-Leu-Xaa-Ile-Ser (position 149–157), Gln-Xaa-Xaa-Xaa-Leu (position 161–165); and Lys-Thr-Gly (position 201–203).

The predicted amino acid sequence of OXA-42 (from strain 1902a) is almost identical to the gene sequenced by the Sanger Institute (from strain K96243) with one amino acid difference. When OXA-42 was compared with other β-lactamases in the EMBL database, this protein displayed high homology with several oxacillin-hydrolysing β-lactamases such as OXA-22 from *Ralstonia pickettii*[18] (51% identity); AsbB1, AmpS and AmpH, the class D enzymes from *Aeromonas jandaei*,[19] *Aeromonas veronii*[20] and *Aeromonas hydrophila*,[15] respectively (45% identities); OXA-18 from *Pseudomonas aeruginosa*[21] (43% identity); and OXA-9 from *Klebsiella pneumoniae* encoded on multiresistant transposon Tn*1311*[22] (41% identity).

The nucleotide sequence of the amplified class D β-lactamase gene from *B. pseudomallei* strain E15 was also determined (EMBL accession number AJ488303). The deduced amino acid sequence, designated OXA-43, is virtually identical to that of OXA-42, differing by two amino acids. Figure 2 presents the alignment of OXA-42 and OXA-43 with the deduced amino acid sequence of the class D β-lactamase gene from *B. pseudomallei* strain K96243, OXA-22, AsbB1, AmpH, OXA-18 and OXA-9.

Prevalence of class D β-lactamase genes in B. pseudomallei

Eleven *B. pseudomallei* strains were investigated for the presence of class D β-lactamase genes. These strains were isolated from Thailand (1901a, 1921a), Vietnam (52.239, 56.91, A203, 52.238, 59.6, A202, 52.237, 55.135) and Australia (60.66) (Table 1). Chromosomal DNAs were isolated and used as templates in PCR amplification. The oligonucleotide primers and PCR conditions were exactly the same as those for strains E15 and 1902a. Strains 1902a and E15 were also included in this amplification as positive controls. The PCR products obtained from eight strains (except 60.66, 52.238 and 52.237) were ~900 bp in length, which corresponded to the predicted PCR product size, regardless of the geographical origin and isolation time (Figure 3 and Table 1). The PCR products were sequenced at both ends to verify that these PCR products were the class D β-lactamase genes (data not shown).

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         10         20         30         40         50         60
CCCACTCTTTGCGGAACAACAATGAAATTCCGACACGCGCTGTCGAGCGCATTCGTTTTG
                        M  K  F  R  H  A  L  S  S  A  F  V  L
         70         80         90        100        110        120
CTGGGTTGCATCGCCGCGTCGGCGCATGCGAAGACGATCTGCACGGCGATCGCCGATGCG
 L  G  C  I  A  A  S  A  H  A  K  T  I  C  T  A  I  A  D  A
        130        140        150        160        170        180
GGCACGGGCAAGCTGCTGGTGCAGGACGGCGATTGCGGCCGCCGCGCATCGCCCGCGTCG
 G  T  G  K  L  L  V  Q  D  G  D  C  G  R  R  A  S  P  A  S
        190        200        210        220        230        240
ACGTTCAAGATCGCGATCAGCCTGATGGGCTACGACGCAGGCTTCCTGCGCAACGAGCAT
 T  F  K  I  A  I  S  L  M  G  Y  D  A  G  F  L  R  N  E  H
        250        260        270        280        290        300
GACCCGGTGCTGCCGTATCGCGACAGTTACATCGCGTGGGGTGGCGAAGCATGGAAGCAG
 D  P  V  L  P  Y  R  D  S  Y  I  A  W  G  G  E  A  W  K  Q
        310        320        330        340        350        360
CCGACCGATCCGACGCGCTGGCTCAAGTATTCGGTCGTGTGGTATTCGCAGCAGGTGGCG
 P  T  D  P  T  R  W  L  K  Y  S  V  V  W  Y  S  Q  Q  V  A
        370        380        390        400        410        420
CACCATCTCGGCGCGCAGCGCTTCGCGCAGTATGCGAAGGCGTTCGGCTACGGCAATGCG
 H  H  L  G  A  Q  R  F  A  Q  Y  A  K  A  F  G  Y  G  N  A
        430        440        450        460        470        480
GACGTGTCCGGCGATCCCGGCCAGAACAACGGCCTCGATCGCGCGTGGATCGGCTCGTCG
 D  V  S  G  D  P  G  Q  N  N  G  L  D  R  A  W  I  G  S  S
        490        500        510        520        530        540
CTGCAGATCTCGCCGCTCGAACAATTGGAATTCCTCGGCAAGATGCTCAATCGCAAGCTG
 L  Q  I  S  P  L  E  Q  L  E  F  L  G  K  M  L  N  R  K  L
        550        560        570        580        590        600
CCCGTGTCGCCCACAGCCGTCGACATGACGGAGCGGATCGTCGAATCGACGACGCTTGCC
 P  V  S  P  T  A  V  D  M  T  E  R  I  V  E  S  T  T  L  A
        610        620        630        640        650        660
GACGGAACGGTGGTGCACGGCAAGACCGGCGTGTCCTATCCGCTGCTGGCCGACGGCACA
 D  G  T  V  V  H  G  K  T  G  V  S  Y  P  L  L  A  D  G  T
        670        680        690        700        710        720
CGCGACTGGGCGCGTGGATCCGGCTGGTTCGTCGGCTGGATCGTGCGTGGCAATCAGACG
 R  D  W  A  R  G  S  G  W  F  V  G  W  I  V  R  G  N  Q  T
        730        740        750        760        770        780
CTGGTGTTCGCGCGCCTCACGCAGGACGAGCGCAAGCAGCCCGTTTCAGCCGGCATACGG
 L  V  F  A  R  L  T  Q  D  E  R  K  Q  P  V  S  A  G  I  R
        790        800        810        820        830        840
ACGCGCGAGGCCTTCCTGCGCGACTTGCCCCGGCTTCTCGCCGCGCGCTGAGCTACTGCC
 T  R  E  A  F  L  R  D  L  P  R  L  L  A  A  R  *
        850        860        870        880
GCCGTCCGCGCAGCCGCGCGACAGGGCGACGTA
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Figure 1. Nucleotide and deduced amino acid sequences of class D β-lactamase from *B. pseudomallei* strain 1902a. Bold letters represent the conserved region of class D β-lactamase. The putative ribosome binding site is underlined.

β-Lactam susceptibilities and β-lactamase activities of the E. coli BL21(DE3) pLysS carrying either bla_{OXA-42} or bla_{OXA-43}

The 900 bp bla_{OXA-42} and bla_{OXA-43} products from *B. pseudomallei* 1902a and E15 parent strains were ligated directly into pCR T7/CT-TOPO and transformed into *E. coli* TOP10F′ as described in Materials and methods. One clone for each strain was selected for further study. The recombinant plasmids were extracted and transformed into *E. coli* BL21(DE3) pLysS.

β-Lactam susceptibilities and β-lactamase (prepared by periplasmic extraction) activities of the *E. coli* BL21(DE3) pLysS carrying the *B. pseudomallei* class D β-lactamase genes were determined. The results are shown in Table 4. Extracts of *E. coli* BL21(DE3) pLysS carrying bla_{OXA-42} or bla_{OXA-43} displayed oxacillinase activity, which was not found in extracts of *E. coli* without plasmid or *E. coli* (pUC18). In contrast, *E. coli* BL21(DE3) pLysS carrying bla_{OXA-42} or bla_{OXA-43} did not show any resistance to ceftazidime or imipenem, and ceftazidime or imipenem hydrolytic activity was not detected in cell extracts.

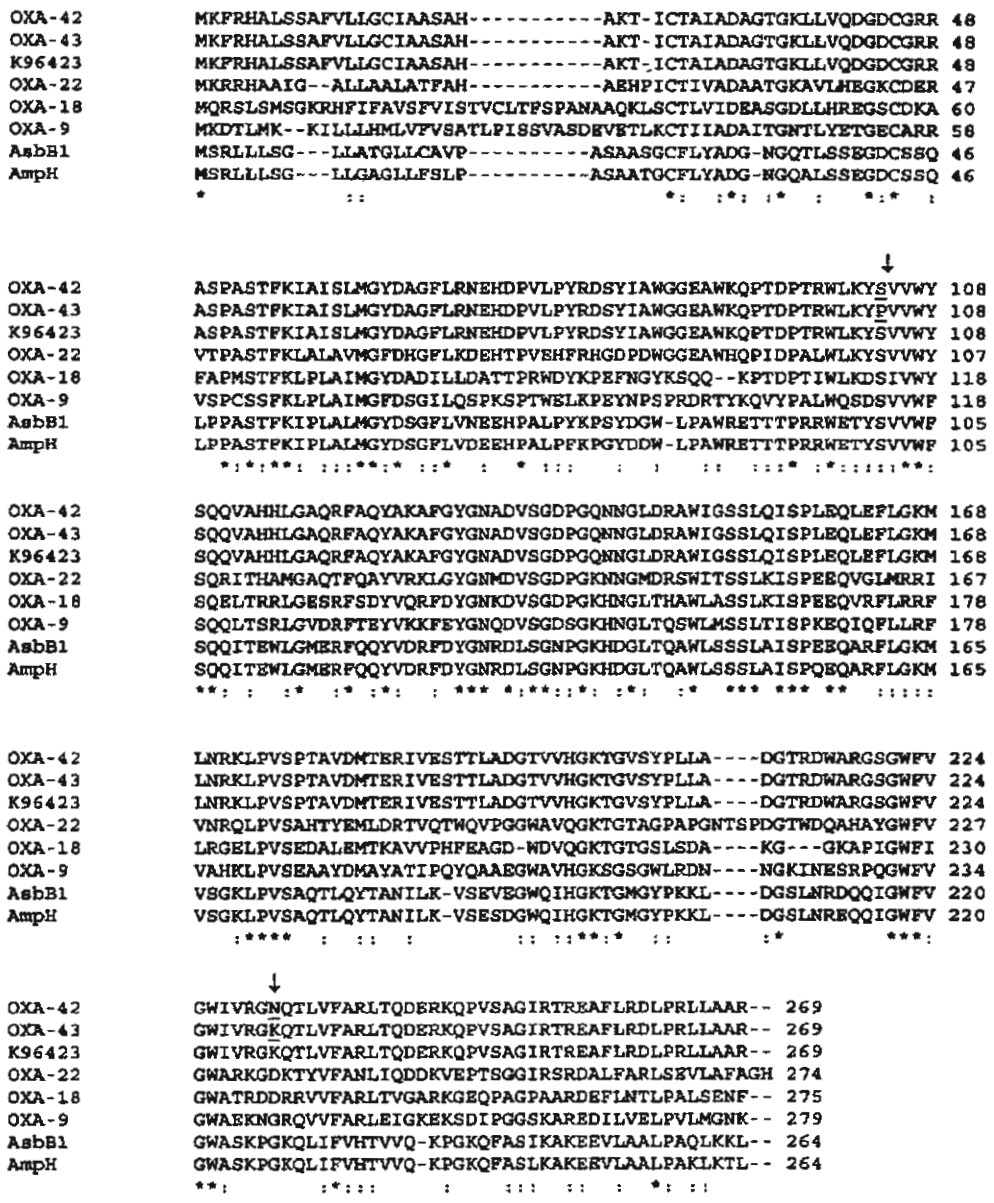

Figure 2. Alignment of class D β-lactamase from *B. pseudomallei* 1902a and E15, OXA-42 and OXA-43, respectively, with the deduced amino acid sequence of the class D gene from *B. pseudomallei* strain K96243 and other related oxacillinases, such as OXA-22,[17] OXA-18,[20] OXA-9,[21] AsbB1[18] and AmpH.[15] The two amino acid differences between OXA-42 and OXA-43 are underlined and indicated by '↓'. Amino acid sequences that are found in all eight class D β-lactamases are indicated by an asterisk. ':' indicates an amino acid that is found in at least five proteins.

Expression of class D β-lactamase in B. pseudomallei E15, parent and ceftazidime-resistant mutant strains

To examine whether the class D β-lactamase is overexpressed in ceftazidime-resistant mutants, RT–PCR was performed on one parent strain and its resistant mutant progeny. *B. pseudomallei* strain E15 was chosen for this study because it was isolated from soil and hence is unlikely to have been exposed to β-lactams at growth inhibitory concentrations. Total RNAs were isolated and quantified. Primers used in cDNA synthesis and RT–PCR are described in Materials and methods. The RNA from both the parent and the ceftazidime-resistant mutant strains was used to synthesize cDNAs, which were further used as templates in amplification. The predicted PCR product is 435 bp. The results showed clearly that the expression of the class D β-lactamase gene is increased significantly

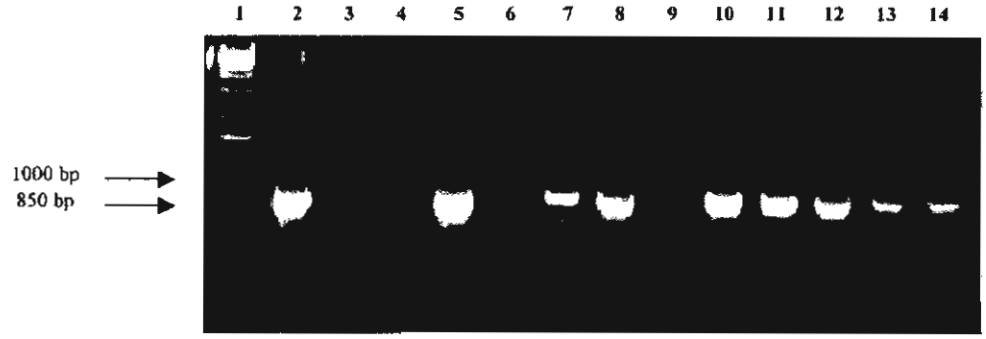

Figure 3. One percent agarose gel electrophoresis of amplification of class D β-lactamase from *B. pseudomallei*. Lane 1, 1 kb plus DNA ladder; lanes 2–14, class D PCR products from *B. pseudomallei* strains 52.239, 56.91, 60.66, A203, 52.238, 59.6, A202, 52.237, 55.135, 1901a, 1902a, 1921a and E15, respectively.

Table 4. β-Lactam susceptibilities and β-lactamase activities of *E. coli* BL21(DE3) pLysS carrying either the bla_{OXA-42} or bla_{OXA-43} gene from *B. pseudomallei*

	MICs (mg/L)			Specific activities[a]		
Strains	CAZ	IPM	OXA	CAZ	IPM	OXA
BL21(DE3) pLysS	<0.125	0.5	256	ND	ND	ND
BL21(DE3) pLysS (pUC18)	<0.125	0.5	512	ND	ND	371 ± 0
BL21(DE3) pLysS (bla_{OXA-42})[b]	<0.125	0.5	512	ND	ND	18 707 ± 1062
BL21(DE3) pLysS (bla_{OXA-43})[b]	<0.125	0.5	512	ND	ND	1432 ± 130

CAZ, ceftazidime; IPM, imipenem; OXA, oxacillin; ND, no detectable activity.
[a]Nanomoles of substrate hydrolysed per minute per milligram of protein.
[b]BL21(DE3) pLysS (bla_{OXA-42}) and BL21(DE3) pLysS (bla_{OXA-43}) are *E. coli* carrying the oxacillinase genes from *B. pseudomallei* strains 1902a and E15, respectively.

in the ceftazidime-resistant mutant compared with the parent strain (Figure 4). This experiment was repeated three times and the results were consistent.

Discussion

β-Lactam resistance poses a potential problem in the treatment of *B. pseudomallei* infection. β-Lactam resistance can emerge in several ways, but the most important mechanism is the production of chromosomally mediated β-lactamase(s). However, mutants in which β-lactamase(s) are produced constitutively at high levels can occur naturally. In this study, ceftazidime-resistant mutants of *B. pseudomallei* were selected at high frequencies. This indicates that if *B. pseudomallei* is exposed repeatedly to ceftazidime, there exists the possibility that resistance will emerge rapidly.

Many ceftazidimases are plasmid encoded, arising as a result of mutations in TEM-, SHV- or the OXA-type β-lactamases, leading to the extended-spectrum β-lactamases (ESBLs). Livermore *et al.*[9] reported β-lactamases from *B. pseudomallei* that were strongly active against many cephalosporins, but not ceftazidime. However, specific ceftazidimase activity in *B. pseudomallei* was reported by Godfrey *et al.*[10] The 25 *B. pseudomallei* tested in this study displayed β-lactamase activities against different β-lactams. The ability to hydrolyse ampicillin or cefalothin was not surprising, but the finding that 48% of *B. pseudomallei* strains tested possess the ability to produce ceftazidimase activity is worrying, as ceftazidime is the antibiotic of choice for the treatment of melioidosis.

The ability of bacteria to hydrolyse imipenem is often mediated by a metallo-β-lactamase, although some serine enzymes have been reported to be able to hydrolyse carbapenems.[6,23] Increased imipenemase activity (more than two-fold) in the *B. pseudomallei* mutant strains was found in 40% of those tested in this study. There is no direct evidence of a metallo-β-lactamase gene in the *B. pseudomallei* genome currently being sequenced, but genome sequencing has revealed the presence of class A, C and D β-lactamase genes. In *P. aeruginosa*, variants of class D β-lactamases are recognized increasingly as being significant in resistance to cef-

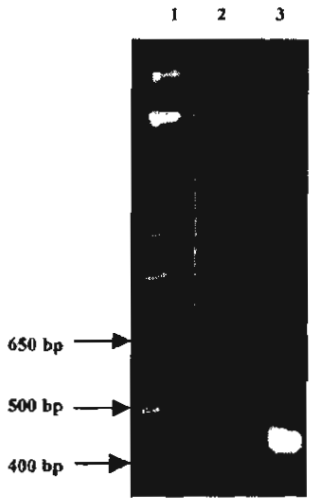

Figure 4. Expression of class D β-lactamases of *B. pseudomallei* E15, parent and mutant strains, by RT–PCR. Lane 1, 1 kb plus DNA ladder; lane 2, amplified product from E15 parent strain; lane 3, amplified product from E15 ceftazidime-resistant mutant (see Materials and methods for more details).

tazidime, i.e. OXA-15,[24] OXA-18[21] and OXA-28.[25] Some OXA-type enzymes have also been reported to hydrolyse carbapenems, e.g. OXA-23,[26] OXA-24,[7] OXA-25, OXA-26 and OXA-27.[8] It was therefore decided to clone and sequence the class D β-lactamase genes from *B. pseudomallei* strains 1902a and E15 and measure their substrate profiles to estimate the role of these enzymes in resistance to imipenem and ceftazidime.

The amino acid sequences of the two *B. pseudomallei* class D β-lactamases, OXA-42 and OXA-43, are almost identical and show significant homology to chromosomally encoded oxacillinases from *R. pickettii* and *Aeromonas* spp. The presence of class D β-lactamase seems to be ubiquitous among *B. pseudomallei* strains, as the geographical origins of these strains were diverse, and the organisms were isolated at different times (one was isolated 55 years ago). Of considerable interest, strain E15 possesses the class D β-lactamase gene but was isolated from a rice field and has never been exposed to β-lactams at therapeutic concentrations. These results support the possibility that the function of β-lactamases is not only to inactivate β-lactams but may also play some other role in cell physiology. If the β-lactamases are involved in crucial cellular functions, then the genes will be maintained in these organisms.

The expression of bla_{OXA-43} is increased in the ceftazidime-resistant *B. pseudomallei* E15 mutant compared with its parent strain, as shown by RT–PCR. However, the results of β-lactamase assays of extracts of *E. coli* carrying either bla_{OXA-42} or bla_{OXA-43} showed no detectable activities against ceftazidime or imipenem, so it is unlikely that overexpression of the class D enzyme is the reason for ceftazidime hydrolytic activity seen in some ceftazidime-resistant *B. pseudomallei* mutants. It should be noted, however, that there have been reports of oxacillinases associated with increases in MIC of ceftazidime up to 128 mg/L, even though they possess no detectable ceftazidimase activity, e.g OXA-16 from *P. aeruginosa* strains 906 and 961,[27] and OXA-17 from *P. aeruginosa* strains 871 and 873.[28] More commonly, however, resistance to third-generation cephalosporins is caused by the production of class A ESBLs[29,30] or the overexpression of class C β-lactamases.[31] Class A and C β-lactamases have already been shown to be involved in β-lactam resistance in *B. pseudomallei*,[32,33] so ceftazidime resistance may be caused by multiple β-lactamases. Their relative roles now need to be elucidated.

The production of multiple β-lactamases has been reported from many bacterial species. For example, expression of three β-lactamases in *Aeromonas* spp. is co-ordinated despite the genes being unlinked.[15,16,19] In the case of *B. pseudomallei*, the observed activities against different β-lactam substrates as well as the results from genome sequencing suggest the presence of multiple β-lactamases. Whether the expression of these enzymes is co-ordinated or not has yet to be determined, but the finding that the class D β-lactamase is overexpressed in mutants selected for resistance to a β-lactam most likely to be hydrolysed by one of the host's other two β-lactamases does suggest some co-ordination of β-lactamase expression.

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