รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาสรีรวิทยาและพันธุศาสตร์ของ *Bacillus thuringiensis*

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วันที่ 24 พฤศจิกายน 2554

สัญญาเลขที่ RTA/05/2540

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัย ขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยที่ให้ทุนสนับสนุนโครงการวิจัยเรื่อง “การศึกษาสรีรวิทยาและพันธุศาสตร์ของ *Bacillus thuringiensis*” ภายใต้ทุนประเภท ทุนสนับสนุนกลุ่มวิจัย-เมธีวิจัยอาวุโส สกว. ในระหว่างเดือน ธันวาคน 2540 ถึงเดือน พฤศจิกายน 2543 เป็นระยะเวลา 3 ปี เพื่อทำการศึกษาวิจัยจนได้ผลการวิจัยที่เป็นประโยชน์ในหลายรูปแบบ คือผลงานวิจัยในลักษณะที่เป็นองค์ความรู้ โดยตีพิมพ์เผยแพร่ในวารสารวิชาการระดับนานาชาติ และในลักษณะที่เป็นเทคโนโลยีและผลิตภัณฑ์ที่มีการทดสอบในระดับกึ่งอุตสาหกรรมเพื่อเป็นแนวทางในการใช้เทคโนโลยีและการผลิตในเชิงพาณิชย์ต่อไป นองจากนี้โครงการวิจัยนี้ยังมีส่วนเสริมให้มีการพัฒนาบุคลากรวิจัยอีกด้วย ทั้งในการผลิตบัณฑิตระดับปริญญาตรี โทและเอก ตลอดจนการพัฒนากลุ่มวิจัยให้นักวิจัยแต่ละคนมีศักยภาพทั้งในด้านงานวิจัยและงานวิชาการสูงขึ้น

คณะผู้วิจัยขอขอบคุณภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่อำนวยความสะดวกในด้านสถานที่ เครื่องมือ ครุภัณฑ์วิทยาศาสตร์ ตลอดจนสิ่งอำนวยความสะดวกต่างๆ จนกระทั่งทำให้ผลงานวิจัยสามารถดำเนินไปได้อย่างมีประสิทธิภาพและประสิทธิผลยิ่ง

ขอขอบคุณ

คณะผู้วิจัย

บทคัดย่อ

การใช้แบคทีเรียชนิด *Bacillus thuringiensis* เพื่อควบคุมแมลงศัตรูพืชและแมลงพาหะของโรคเป็นหนึ่งในวิธีการควบคุมแมลงโดยขบวนการชีววิธี ซึ่งเป็นที่เชื่อกันว่าเป็นวิธีที่เอื้อต่อสิ่งแวดล้อมมากที่สุดวิธีหนึ่ง เนื่องจากเป็นเชื้อที่มีความจำเพาะสูงและไม่เป็นพิษต่อแมลงหรือสิ่งมีชีวิตอื่นๆ ที่ไม่ใช่เป้าหมาย อย่างไรก็ดีเกษตรกรหรือนักการสาธารณสุขมักไม่ค่อยนิยมใช้ผลิตภัณฑ์ที่มีเชื้อ *B. thuringiensis* ในการควบคุมแมลงศัตรูพืชหรือแมลงพาหะของโรค เนื่องจากผลิตภัณฑ์ที่มีอยู่ในท้องตลาดเป็นผลิตภัณฑ์ที่ล้วนนำเข้าจากต่างประเทศ ซึ่งคุณภาพไม่เหมาะสมกับการ ใช้งานในประเทศไทยที่มีภูมิอากาศแบบร้อนชื้น นอกจากคุณภาพไม่เหมาะสมแล้วยังมีราคาค่อนข้างสูง โดยองค์ประกอบทั้งสองด้านนี้เป็นผลให้ผลิตภัณฑ์ไม่เป็นที่นิยมมากนัก ดังนั้นหากมีการพัฒนาให้เกิดอุตสาหกรรมเพื่อผลิต *B. thuringiensis* ได้ในประเทศไทย ก็จะสามารถแก้ปัญหาเรื่องคุณภาพที่ไม่เหมาะสมและราคาสูงจากการนำเข้าผลิตภัณฑ์จากต่างประเทศได้ อย่างไรก็ดีก่อนที่จะสามารถผลักดันให้เกิดอุตสาหกรรมเพื่อผลิต *B.thuringiensis* ได้นั้นจำเป็นอย่างยิ่งที่จะต้องมีองค์ความรู้พื้นฐานเกี่ยวกับแบคทีเรียชนิด *B. thuringiensis* ก่อน ดังนั้นโครงการวิจัยนี้จึงศึกษาเกี่ยวกับสรีรวิทยาและพันธุศาสตร์ของ *B. thuringiensi* ทำให้ทราบถึงสภาวะที่เหมาะสมในการเพาะเลี้ยงแบคทีรเรียชนิดนี้ ทั้งในลักษณะที่เป็นระบบ batch, fed-batch และ cell-recycle ตลอดจนสามารถหาสูตรอาหารที่เหมาะสมในการเพาะเลี้ยง ซึ่งองค์ความรู้เหล่านี้สามารถนำไปใช้ขยายขนาดการผลิตในระดับกึ่งอุตสาหกรรมและระดับอุตสาหกรรมได้ในที่สุด นอกจากนี้โครงการนี้ยังประสบผลสำเร็จในการคัดเลือกสายพันธุ์ของ *B. thuringiensis* ที่เหมาะสมในการทำลายหนอนแมลงศัตรูพืชได้อย่างมีประสิทธิภาพอีกด้วย เป็นผลให้เกิดการพัฒนาทั้งทางด้านสรีรวิทยาและพันธุศาสตร์อย่างดียิ่ง

นอกจากโครงการนี้จะประสบผลสำเร็จในการพัฒนาเทคโนโลยีในการผลิต *B. thuringiensis* แล้ว ยังสามารถตีพิมพ์บทความเพื่อเผยแพร่ผลงานวิจัยในวารสารนานาชาติจำนวน 6 เรื่อง ตลอดจนได้มีการเผยแพร่ผลงานวิจัยในลักษณะอื่นๆ เช่น การเสนอรายงานในการประชุมวิชาการถึง 23 ครั้ง ผลลัพธ์ที่สำคัญอีกประการหนึ่งของโครงการนี้คือ สามารถพัฒนาบุคลากรจำนวน 7 คน ให้มีศักยภาพทั้งทางด้านการวิจัยและทางด้านวิชาการสูงขึ้นอีกด้วย

คำหลัก: การควบคุมแมลงโดยชีววิธี, *Bacillus thuringiensi*, ผลิตภัณฑ์กึ่งอุตสาหกรรม, สูตรผสมชีวภาพ, อุตสาหกรรมชีวภาพ

Abstract

The use of *Bacillus thuringiensis* as bacterial agent in controlling insect pests and vectors is a more preferred method over those of using chemical agents due to the more environmentally friendly action of the biological control methods. The highly specify of the biological control agents toward targeted insect is the main reason for the advantage in the use of biological control methods. However, due to the poor quality and high price of the imported biological control agents, the method is not very popular with the local farmer and public health personal. The imported products are usually not optimized for the tropical climates of high temperature and high humidity conditions in Thailand. As such, it is highly desirable to develop *B. thuringiensis* based formulation with suitable performance in the more tropical climates. It is envisage that the locally developed products will not only improve the quality of the product but also lead to the reduction in the cost as well. With the aim in promoting commercial scale production of *B. thuringiensis* base biopesticides, this research project was undertaken to study basic physiology and genetics of *B. thuringiensis*. The results obtained through basic research study has lead to development of appropriate media composition for growth of *B. thuringiensis* both at laboratory and pilot scale levels. Results from basic genetic study of *B. thuringiensis* also lead to successful isolation of the most appropriate strains used for controlling insect vectors and pests. Experiments were also conducted to evaluate various methods for cultivation for example, batch, fed-batch, and cell recycle. Research results acquired in this study have lead to the contraction or commercial scale production plant for *B. thuringiensis* based biopesticide products.

Beside success in commercialization of the research results, this study also met the objectives in publishing as many as 6 articles in international research journals, in presenting the works at workshop and academic symposia more than 23 times. Furthermore, the project also lead to the completion of 1 doctoral degree graduates and 6 Master degree graduates. Thus, this research project has demonstrated the capability to accomplish by the research activities as well as in human resource development.

Key words: *Bacillus thuringiensis*, Biocontrol, Biocide formulation, Industrial Production, Pilot scale production

ผลงานที่ได้จากโครงการ การศึกษาสรีรวิทยาและพันธุศาสตร์ของ *Bacillus thuringiensis*

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6. อื่น ๆ

6.1. กำลังเขียน manuscript เพื่อการตีพิมพ์ในวารสาร ดังนี้คือ

6.1.1. Bhumiratana, A. Technology Transfer for a Small and Medium Bio-industry.

6.1.2. Wiwat, C., Jitaleela, C., and Bhumiratana, A. In vivo conjugation between *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *israelensis*, and toxicity of their transconjugant toward *Ades aegypti* larvae.

6.1.3. Duangrat Inthorn. The use of microalgae for color and chemical oxygen demand removal in treated molasses wastewater.

6.1.4. Duangrat Inthorn. N and P removal in wastewater by using *Spirulina platensis*

6.1.5. Duangrat Inthorn. Screening of tolerance microalgae for mercury, cadmium and lead removal.

6.1.6. Inthorn, D., Chanchitpricha, C., and Bhumiratana, A. Screening of microalgae having the ability to decolorize and COD removal in molasses pigment.

6.1.7. Inthorn, D., Chanchitpricha, C., and Bhumiratana, A. Improvement of color and COD removal by the use of Fenton's reagent and ozone.

6.1.8. Inthorn, D., Sidtitoon, N., and Bhumiratana, A. Screening of mercury, cadmium and lead in aqueous solution by the use of microalgae.

6.1.9. Inthorn, D., Sidtitoon, N., and Bhumiratana, A. Removal of mercury, cadmium and lead in aqueous solution by the use of microalgae.

6.1.10. Inthorn, D., Auijirakul, K., and Bhumiratana, A. Use of *Spirulina sp.* in treatment of wastewater from soft drink factory.

6.1.11. Inthorn, D., Sidtitoon, N., and Bhumiratana, A. Adsorption mechanism of heavy metals on cell surface of cyanobacterium *Tolypothrix tenuis*.

6.1.12. Inthorn, D., Sidtitoon, N., and Bhumiratana, A. Removal of lead from aqueous solution by filamentous cyanobacterium *Tolypothrix tenuis* and *Calothrix parientina.*

6.1.13. ดวงรัตน์ อินทร. การใช้สาหร่ายในการกำจัดโลหะหนัก. คาดว่าจะตีพิมพ์ในวารสารวิทยาศาสตร์และเทคโนโลยี.

6.1.14. ดวงรัตน์ อินทร. อันตรายจากการปนเปื้อนของตะกั่วในจานชามที่ใช้กับเตาไมโครเวฟ คาดว่าจะตีพิมพ์ในวารสารการส่งเสริมสุขภาพและอนามัยสิ่งแวดล้อม หรือ วารสารพยาบาลสาธารณสุข.

6.1.15. Chobchuenchom, W., Moungnoi, S., and Bhumiratana, A. Toxicity of Thai indigenous plants extracts to *Pomucea canciliculata* (lamark).

6.1.16. Chobchuenchom, W., and Bhumiratana, A. Effect of *Pseudomonas pluorescens* ct1 on *Pomucea canciliculata* (lamark) histology and enzymes.

6.1.17. Application of fed-batch cultivation for high cell density and spore formation of *Bacillus thuringiensis* var *kurstaki* .

6.1.18. Process optimization for production of *Bacillus thuringiensis* var *kurstaki* endotoxin with high insecticidal activity using fed-batch culture.

7. การถ่ายทอดผลงานวิจัยสู่เชิงพาณิชย์

ผลงานวิจัยนี้ก่อให้เกิดการจัดตั้งบริษัท ที เอฟ ไอ กรีน ไบโอเทค จำกัด (TFI Green Biotech Co., Ltd.) โดยมีกำลังการผลิต *Bacillus thuringiensis* สูตรทำออกสู่ตลาดในเชิงพาณิชย์ได้ตามรายละเอียดในสัญญาการอนุญาตให้ใช้และการถ่ายทอดเทคโนโลยี เรื่อง การผลิตสารชีวินทรีย์ *Bacillus thuringiensis* สูตรน้ำในระดับอุตสาหกรรม ดังแนบ โดยบริษัทมีกำลังการผลิตโดยใช้ Fermentor ขนาดตัวละ 3 ตัน จำนวน 3 เครื่อง ซึ่งบริษัทได้ผลิตผลิตภัณฑ์ออกสู่ท้องตลาดอย่างต่อเนื่อง

<u>บริษัท ทีเอฟไอ กรีนไบโอเทค จำกัด</u>
<u>ตั้งอยู่ เลขที่ 15 หมู่ 17 ต. ท่าผา</u>
<u>อ. บ้านโป่ง จ. ราชบุรี</u>

ภายในโรงงานที่มี Fermentor

ขนาด 3 ตัน จำนวน 3 เครื่อง

บรรจุภัณฑ์ของ *Bacillus thuringiensis* ที่พร้อมจำหน่าย

รายชื่อกลุ่มวิจัย

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ (พ.ศ. 2540)			เมื่อสิ้นสุดโครงการ (พ.ศ. 2543)		
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4. นพ. วิวิทย์ ศมศานติ์	อาจารย์	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. สงขลานครินทร์	ผู้ร่วมวิจัย	อาจารย์	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. สงขลานครินทร์	ยังอยู่ในโครงการ
5. ดร. วิมล ชอบชื่นชม	อาจารย์	ภาควิชาจุลชีววิทยาคลีนิก คณะเทคนิคการแพทย์ ม. รังสิต	ผู้ร่วมวิจัย	ผศ.	ภาควิชาจุลชีววิทยาคลีนิก คณะเทคนิคการแพทย์ ม. รังสิต	ยังอยู่ในโครงการ
6. ดร. ดวงรัตน์ อินทร	อาจารย์	ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ ม. มหิดล	ผู้ร่วมวิจัย	ผศ.	ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ ม. มหิดล	ยังอยู่ในโครงการ
7. Dr. Cornel Verdyun	อาจารย์	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	ผู้ร่วมวิจัย	อาจารย์	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	ไม่ได้อยู่ในโครงการ

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ (พ.ศ. 2540)			เมื่อสิ้นสุดโครงการ (พ.ศ. 2543)		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
8. น.ส. ทิพวรรณ เหลืองแดง	-	ภาควิชาจุลชีววิทยาคลีนิก คณะเทคนิคการแพทย์ ม. รังสิต	ผู้ช่วยวิจัย	-	ภาควิชาจุลชีววิทยาคลีนิก คณะเทคนิคการแพทย์ ม. รังสิต	ยังอยู่ในโครงการ
9. น.ส. เสาวลักษณ์ วิมุตติพงศ์	-	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. สงขลานครินทร์	ผู้ช่วยวิจัย	-	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. สงขลานครินทร์	ยังอยู่ในโครงการ
10. นายนรินทร์ ชนวิทยาสิทธิกุล	-	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	ผู้ช่วยวิจัย	-	-	นักศึกษาปริญญาโท
11. น.ส. สรัญญา ไทยถานันดร์	-	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท	-	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ ม. มหิดล	นักวิจัยในโครงการ
12. น.ส. อาพันธ์ชนิด เทพอวยพร	-	ภาควิชาจุลชีววิทยา คณะเภสัชศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท	-	ภาควิชาจุลชีววิทยา คณะเภสัชศาสตร์ ม. มหิดล	นักวิจัยในโครงการ
13. น.ส. ชื่นจิต ชาญชิตปรีชา	-	ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท	-	ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ ม. มหิดล	กำลังศึกษาต่อต่างประเทศ
14. น.ส. นลิน สิทธิธูรณ์	-	ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท	-	ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ ม. มหิดล	นักวิจัยในโครงการ
15. น.ส. อังคณา เสาวภาคย์หิรัณย์	-	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท	-	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ (พ.ศ. 2540)			เมื่อสิ้นสุดโครงการ (พ.ศ. 2543)		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
16. น.ส. ขัตติยา สอนสนาม	-	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท	-	ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ ม. มหิดล	นักศึกษาปริญญาโท
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CURRENT MICROBIOLOGY Vol. 39 (1999), pp. 134–140

Current Microbiology
An International Journal

Purification and Characterization of Chitinase from *Bacillus circulans* No.4.1

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Received: 19 March 1999 / Accepted: 30 April 1999

Abstract. *Bacillus circulans* No.4.1 produced a high level of chitinase when cells were grown in tryptic soy broth supplemented with 0.3% colloidal chitin at 35°C for 5 days. Purification was carried out by protein precipitation with 80% saturation ammonium sulfate, anion-exchange chromatography with DEAE-Sephacel, and gel filtration with Sephadex G-100, sequentially. The purified enzyme could be demonstrated as a single band on SDS-PAGE, estimated to be 45 kDa. This enzyme could hydrolyze colloidal chitin, purified chitin, glycol chitin, carboxymethyl-chitin (CM-chitin), and 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside [4-MU-$(GlcNAc)_2$]. The optimal conditions for this chitinase were pH 8.0 and 40°C. The isoelectric point of the chitinase was 5.1. The amino acid composition of the purified chitinase was determined. The initial 20 amino acid residues of the N-terminal were found to be alanine (A), proline (P), tryptophan (W), asparagine (N), serine (S), lysine (K), glycine (G), asparagine (N), tyrosine (Y), alanine (A), leucine (L), proline (P), tyrosine (Y), tyrosine (Y), arginine (R), glycine (G), alanine (A), tryptophan (W), alanine (A), and valine (V). Knowledge of these properties of chitinase from *B. circulans* No. 4.1 should be useful in the development of genetically engineered *Bacillus* sp. as biopesticides.

Chitinases are commonly found in a wide range of organisms, including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates [6, 10]. The roles of these chitinases may be divided into several categories; for example, a major role of fungi, crustaceans, and insects is modification of the organisms' structural constituent chitin. The production of chitinases by plants is considered to be a part of their defense mechanism against fungal pathogens. Bacteria produce chitinase to digest chitin, primarily to utilize it as a carbon and energy source [8, 13, 15]. The chitinases are classified as endochitinase, exochitinase (EC.3.2.1.14), β-*N*-acetylglucosaminidase, and chitobiase (EC.3.2.1.30), which degrade chitin and its derivatives. Endochitinase splits the chitin polymer internally, whereas exochitinase releases chitobiose from one end. β-*N*-acetylglucosaminidase releases *N*-acetylglucosamine (NAG) monomers from chitin, while chitobiase hydrolyzes chitobiose to *N*-acetylglucosamine [6]. Enzymatic hydrolysis of chitin to its constituent monomer is performed by the binary chitinase system [7]. Chitinases are constituents of several microbial species; some of the best known include the *Aeromonas*, *Serratia*, *Myxobacter*, *Vibrio*, *Streptomyces*, and *Bacillus* genera. Strains of *Serratia marcescens*, *Bacillus*, and *Vibrio* have been shown to produce a high level of chitinolytic enzymes [5]. Cody et al. studied the distribution of chitinase and chitobiase in strains of *Bacillus* and found that *Bacillus licheniformis* X-70 produced a thermostable chitinase [4, 18]. *B. circulans* WL-12 is one of the bacteria that secretes chitinases into the culture medium [24]. When the bacterium was grown in a medium containing chitin as an inducer substrate, six distinct chitinases were detected in the cultural supernatant. Chitinase A1 showed strong affinity to chitin and was suggested to play a major role in the degradation of chitin in the chitinase system of *B. circulans* WL-12 [24].

Our previous study demonstrated that *B. circulans* No.4.1 could degrade chitin at high pH (pH 6–12) and that a partially purified chitinase from this organism

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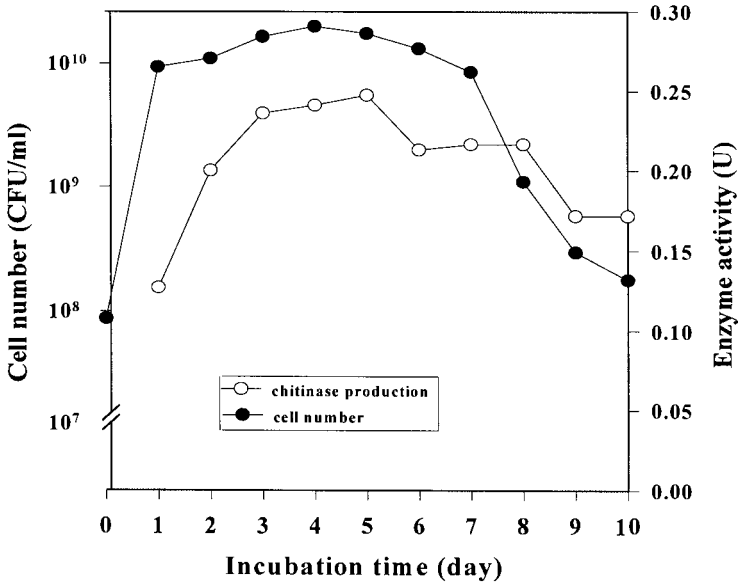

Fig. 1. Time course for production of chitinase by *B. circulans* No.4.1 grown in TSB supplemented with 0.3% colloidal chitin at 35°C.

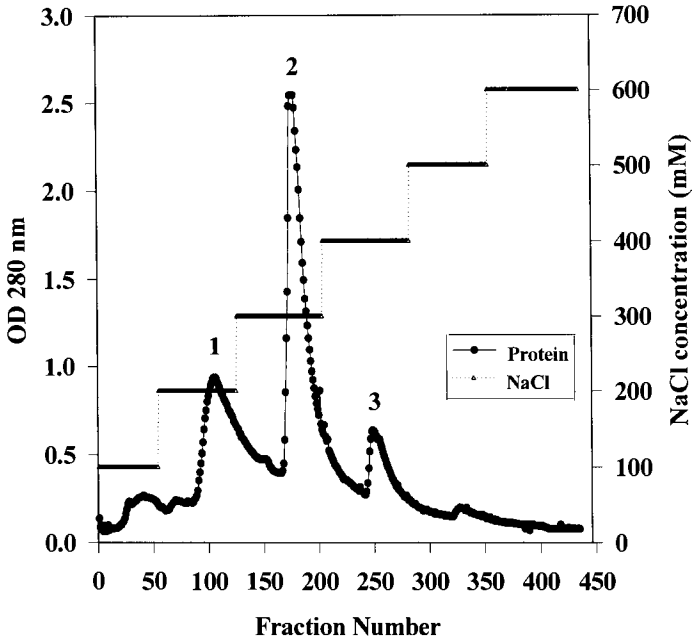

Fig. 2. Elution profile of chitinase from DEAE-Sephacel column chromatography. The protein was eluted stepwise with 10 mM Tris-HCl containing 0.1 to 0.6 M NaCl. Peak No. 3 possessed chitinase activity.

could enhance the toxicity of *Bacillus thuringiensis* subsp. *kurstaki* towards diamondback moth larvae [26]. Thus, the toxicity of *B. thuringiensis* toward target insects might be increased by the introduction of a chitinase-encoding gene.

The objective of this study was to purify the extracellular chitinase from *B. circulans* No.4.1 and characterize its enzymatic properties. The results of this investigation will be useful in the creation of genetically engineered microorganisms that possess high chitinase activity for use as biological control agents for insect pests.

Materials and Methods

Chemicals. Sephadex G-100, 3-cyclohexylamino-1-propanesulfonic acid, *N*-acetylglucosamine, bovine serum albumin, purified chitin, carboxy methyl-chitin, 4-methylumbelliferyl-β-D-*N*, *N′*-diacetylchitobioside [4-MU-$(GlcNAc)_2$], ammonium sulfate, standard molecular weight markers, and isoelectric point (p*I*) standard proteins (p*I* 4.45–9.6) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). DEAE-Sephacel resin was purchased from Pharmacia LKB (Uppsala, Sweden). Chromatography column and protein assay dye-reagent concentrate were obtained from Bio-Rad (Richmond, CA). Mini ProBlott™ was from Applied Biosystems (Foster City, CA).

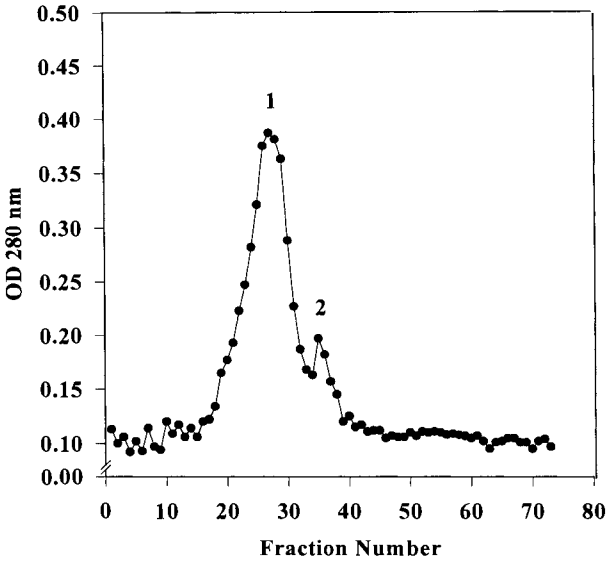

Fig. 3. Elution profile of chitinase from Sephadex G-100 column chromatography. Pooled chitinase from peak No. 3 of DEAE-Sephacel was concentrated and loaded onto the column. The protein was eluted with 10 mM Tris-HCl buffer. Peak No. 1 possessed chitinase activity.

Preparation of colloidal chitin. Colloidal chitin was prepared from commercial chitin by the method of Roberts and Selitrennikoff [15]. Twenty grams of chitin powder was added slowly into 350 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 liters of ice-cold 95% ethanol with rapid stirring and kept overnight at −35°C. The precipitate was collected by centrifugation at 5000 *g* for 20 min at 4°C (Beckmann). The precipitate was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0), then dried by lyophilization.

Organism and culture conditions. *B. circulans* No.4.1 was isolated from soil in Thailand [26], and a pure culture was maintained in 15% glycerol at −80°C. A single colony was inoculated into 10 ml of tryptic soy broth (TSB) supplemented with 0.3% colloidal chitin and incubated at 35°C for 48 h on a rotary shaker (200 rpm). Five milliliters of preculture was inoculated into a 1.5-L Erlenmeyer flask containing 500 ml of the same medium and further incubated for 5 days. The culture broth was centrifuged at 4°C for 20 min at 9000 *g*. The supernatant was collected for purification of the enzyme.

Determination of protein. Protein was monitored by measuring the absorbance at 280 nm during chromatographic separation. Protein was also determined by the method described by Bradford [3], with the Bio-Rad protein assay dye reagent and BSA as a reference protein.

Determination of chitinase activity

Colorimetric method. Chitinase activity was determined by measuring the amount of the reducing end group, NAG, degraded from colloidal chitin, as described by Ueda and Arai [21]. The assay mixture consisted of 0.1 ml of enzyme solution, 0.1 ml of 0.3% colloidal chitin, and 0.2 ml of 0.1 M McIlvaine buffer, pH 6.0. After incubation at 35°C for 25 min, the reaction was terminated by heating in boiling water for 15 min. Subsequently, 2.0 ml of 1.5 mM potassium ferricyanide reagent was added, and the clear solution was measured immediately by a spectrophotometer at 420 nm. The activity was calculated from a standard curve obtained from known concentrations of NAG (0–0.15 mg).

Table 1. Purification of chitinase from *Bacillus circulans* No. 4.1

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme (supernatant)	871.15	165.9	0.19	1.00	100.00
80% Ammonium sulfate precipitation	145.69	36.78	0.25	1.32	22.17
DEAE-Sephacel column	33.43	11.55	0.35	1.84	6.96
Sephadex G-100 column	0.72	0.71	0.99	5.21	0.43

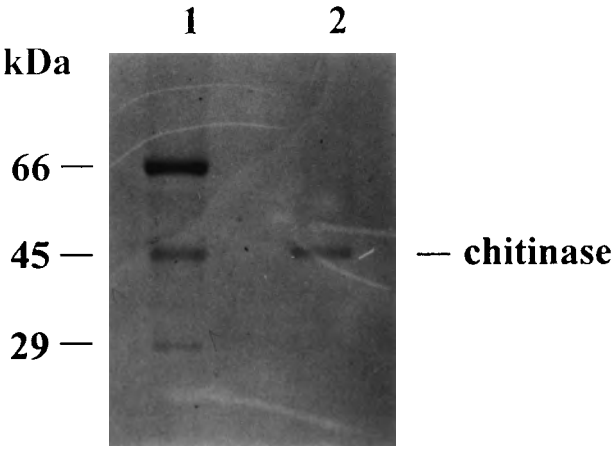

Fig. 4. Molecular weight determination of chitinase by denaturing gel electrophoresis. Lane 1: molecular weight marker (bovine carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa). Lane 2: pooled fraction obtained from Sephadex G-100 column.

One unit of activity was defined as the amount of enzyme that liberated 1 μmol of NAG per min during these conditions. Negative control tubes contained all components except substrate, and blanks contained all components except the enzyme.

Purification of chitinase. Unless otherwise stated, all purification steps were carried out at 4°C.

Ammonium sulfate precipitation. The culture filtrate was fractionated with ammonium sulfate of 80% saturation. The protein precipitate was obtained by centrifugation at 9000 *g* for 20 min. The precipitate was dissolved in small volumes of sterile distilled water. The solution was dialyzed overnight at 4°C against four changes of 4 liters each of distilled water containing 1 mM EDTA. The dialysate was collected and used for further purification.

Anion exchange chromatography. DEAE-Sephacel was prepared by the method of the Pharmacia Company. The enzyme was dialyzed against 10 mM Tris-HCl buffer (pH 8.2). The dialyzed solution was applied to a DEAE-Sephacel column (2.5 × 50 cm) and equilibrated with the same buffer. A flow rate of 0.15 ml/min was maintained. The unbound protein was washed with 2 bed volumes of starting buffer. The enzyme was eluted stepwise with 0.1–0.6 M NaCl in 10 mM Tris-HCl buffer (pH 8.2). Fractions of 5.0 ml were collected. Protein profile was monitored by measuring the absorbance at 280 nm. The fractions that possessed chitinase activity were pooled, concentrated by ultrafiltration with

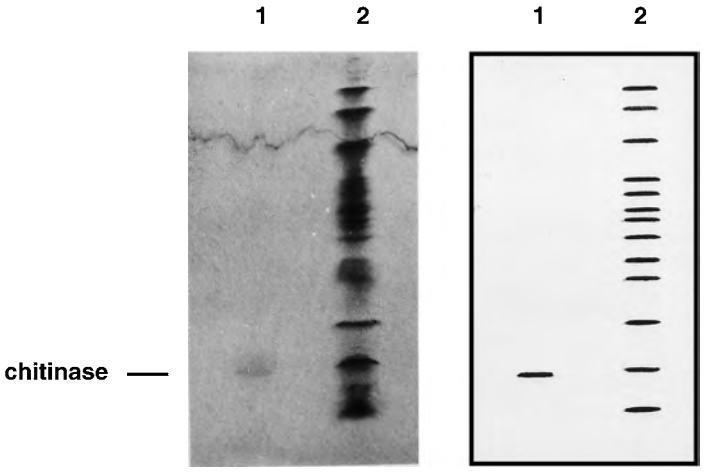

Fig. 5. IEF pattern of chitinase from *B. circulans* No.4.1. Lane 1: Purified chitinase. Lane 2: IEF standard marker.

Amicon PM-10 membrane (Grace Company) to give a final volume of 5.0 ml, and stored at −20°C for further purification by gel filtration.

Gel filtration chromatography. Sephadex G-100 was prepared by the method of the Pharmacia Company. Sephadex G-100 was swollen in 10 mM Tris-HCl buffer, pH 8.2 (5.0 mg/ml) for 5 h at 90°C. The swollen gel was degassed before being packed in a column (1.5 × 50 cm). The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.2) at a constant flow rate of 0.05 ml/min. The 1.0 ml of concentrated enzyme that was obtained from the DEAE-Sephacel column was applied to the column and eluted with the same buffer. Fractions of 3.0 ml were collected at a flow rate of 0.05 ml/min. Protein profile was monitored by measuring the absorbance at 280 nm. The fractions that possessed chitinase activity were pooled, concentrated by ultrafiltration with Amicon PM-10 membrane (Grace Company), and then dialyzed overnight against 10 mM Tris-HCl buffer (pH 8.2). The concentrated enzyme was kept at −20°C until use.

Determination of molecular weight. SDS-PAGE was performed according to the method described by Laemmli [12] with the Bio-Rad Mini Protean II apparatus, a 10% acrylamide separating gel, and 3.75% acrylamide for stacking gel containing 0.1% SDS. The proteins were stained with Coomassie brilliant blue R-250.

Optimal pH of chitinase. The chitinase activity was measured at various pHs by a colorimetric method, using colloidal chitin as a substrate. The enzyme was preincubated in 0.1 M McIlvaine buffer at pH 3–7, 50 mM Tris-HCl buffer at pH 8.0, or 50 mM glycine-NaOH buffer at pH 9–12.

Optimal temperature of chitinase. The enzyme was preincubated in 50 mM Tris-HCl buffer (pH 8.0) at different temperatures from 10° to 70°C for 25 min. The reaction was followed by the colorimetric method, with colloidal chitin as a substrate.

Substrate specificity of chitinase. The enzyme was assayed with chitin and its derivatives as substrate in 50 mM Tris-HCl buffer (pH 8.0) for 25 min at 40°C, with the colorimetric method.

Isoelectric focusing (IEF). Isoelectric focusing was performed with Phastsystem (Pharmacia Company). A phastgel IEF 3–9 was placed in the Phastsystem separation unit. Samples of 1.0 µl were loaded with a Pharmacia sample applicator (model 8/1). Electrophoresis was performed for 30 min, and the temperature of the cooling bed was 15°C. The gel was stained in the Phastsystem development unit with the Pharmacia Coomassie blue staining method. Bio-Rad's IEF standards, a mixture of nine natural proteins with isoelectric point (p*I*s) ranging from 4.45 to 9.6, were used.

Table 2. Amino acid composition of chitinase from *B. circulans* No. 4.1

Amino acid	% Amino acid
Aspartic acid	17.25
Threonine	4.67
Serine	4.32
Glutamic acid	14.02
Proline	7.94
Glycine	11.43
Alanine	8.36
Cysteic acid	0.83
Valine	6.63
Methionine	0.08
Isoleucine	4.27
Leucine	5.78
Tyrosine	2.50
Phenylalanine	2.95
Histidine	1.83
Lysine	4.01
Trytophan	0.05
Arginine	3.08
Total	100.00

Amino acid composition. Amino acid analysis of chitinase was measured by the method described by the Beckman Company. The enzyme solution was dried by a SC100A Speedvac Plus (Savant). The samples were hydrolyzed with 6 M HCl, 4 M methanesulfonic acid for tryptophan in evacuated tubes, and the tubes were sealed under vacuum at 110°C for 22 h by the Waters Pico-Tag Workstation. For analysis of cystine and methionine, the samples were hydrolyzed with performic acid reagent for 30 min before the addition of 6 M HCl. The resulting amino acid compositions were analyzed on a Na high-performance column and calculated by an amino acid analyzer (Beckman System 6300).

N-terminal amino acid sequencing. After SDS-PAGE, the gel was subjected to electroblotting with Mini Trans-Blot Electrophoresis

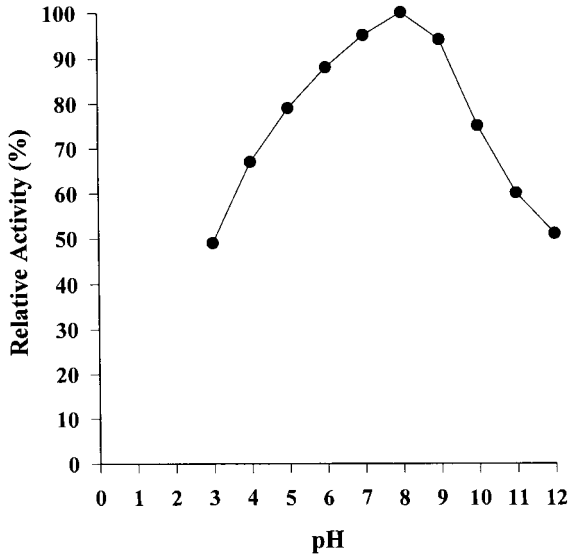

Fig. 6. The pH profile of chitinase from *B. circulans* No.4.1.

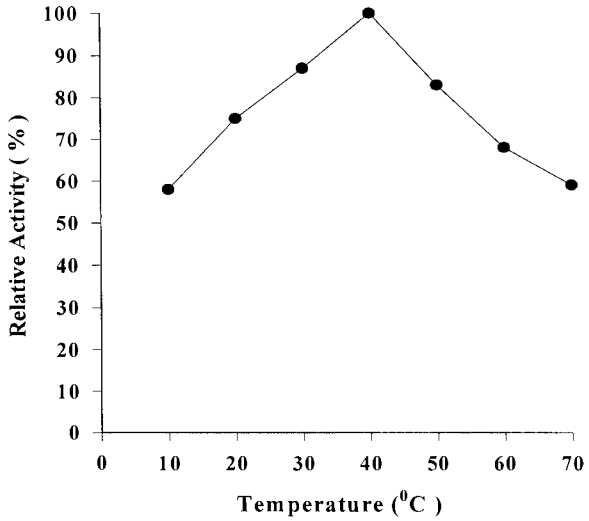

Fig. 7. The temperature profile of chitinase from *B. circulans* No. 4.1.

Table 3. Substrate specificity of chitinase from *B. circulans* No. 4.1

Substrate	Specific activity (U/mg)
Purified chitin	0.15
Colloidal chitin	0.16
Glycol chitin	0.24
CM-chitin	0.12
4-MU-$(GlcNAc)_2$	0.16

Transfer Cell (Bio-Rad), as described by the Applied Biosystem Company. The immobilization membrane (ProBlott™) was used as a transfer membrane. After electroblotting, the protein bands in the membrane were visualized by Coomassie blue staining. The membrane was then destained with 50% methanol until protein bands appeared and rinsed extensively with deionized water. The bands were excised and stored at −20°C until sequencing.

Amino acid sequencing was performed by the method described by Applied Biosystems Company. Automated cycles of Edman degradation were performed by a gas phase protein sequencer (Applied Biosystems model 476A), and phenylthiohydantoin (PTH) amino acid derivatives were automatically identified by the data analysis apparatus (Applied Biosystems model 610A). The membrane sample from electroblotting was dried by a SC100A Speedvac Plus and placed into the Blot cartridge. The resulting PTH amino acids were analyzed on an Applied Biosystems PTH-C18 reversed-phase cartridge column.

Results

Time course of production of chitinase. *B. circulans* No.4.1 showed maximal specific activity of extracellular chitinase (0.9 unit/mg protein) when the culture reached a cell density of 0.712 (OD 580 nm) or equivalent to 1.75×10^{10} CFU/ml on the fifth day of culture (Fig. 1).

Purification of chitinase from *B. circulans* No. 4.1. *B. circulans* No.4.1 released chitinase into the culture broth. Purification by 80% ammonium sulfate precipitation followed by DEAE-Sephacel chromatography resulted in three protein peaks (Fig. 2). The 0.3–0.4 *M* NaCl fraction contained chitinase activity. The final specific activity of chitinase in this step was 0.35 unit/mg protein with a purification factor of 1.84. Gel filtration produced a major peak that possessed chitinase activity (Fig. 3). The final specific activity of purified chitinase in this step was 0.99 unit/mg protein with a purification factor of 5.21. Quantitative results obtained from the purification steps are shown in Table 1. The protein obtained from fractions 19 to 41 was subjected to SDS-PAGE: a single band of chitinase was observed at 45 kDa (Fig. 4).

Isoelectric point of chitinase. The purified chitinase, which showed a single band on SDS-PAGE, was subjected to iso-electrofocusing. The p*I* of chitinase was estimated to be 5.1 as shown in Fig. 5.

Amino acid composition and N-terminal sequence of chitinase. The amino acid composition of the purified chitinase showed that the most abundant amino acid residues were aspartic acid (17.25%), glutamic acid (14.02%), and glycine (11.43%) (Table 2). The N-terminal sequence was found to be A-P-W-N-S-K-G-N-Y-A-L-P-Y-Y-R-G-A-W-A-V.

Optimal conditions for chitinase. The enzyme could hydrolyze colloidal chitin at all tested pHs. The optimal pH was 8.0 (Fig. 6). Fifty percent of enzymatic activity was lost at extremely low (3) or high (12) pH. The optimal temperature was 40°C, above which chitinase activity decreased (Fig. 7).

Substrate specificity. The chitinase could hydrolyze purified chitin, colloidal chitin, and all soluble derivatives

Table 4. Comparison of 20 N-terminal amino acid residues of chitinase from *B. circulans* No. 4.1 to other microorganisms

Microorganism	Amino acid residue	Reference
B. circulans WL-12 chiA1	A D S Y L I V G Y Y P S W A A Y G R N Y	26
S. marcescens QMB 1466	A P G L P T I A W G N T L F A I V E V D	11
Altermonas sp. strain O-7	A P S T P T L D W Q P Q Q Y S L V E V N	20
Bacillus sp. strain MH1	A T P A T A T Y S T D S D W E T G F Q Q	17
B. circulans No. 4.1	A P W N S K G N Y A L P Y Y R G A W A V	This study

tested, CM-chitin, glycol chitin, and 4-MU-$(GlcNAc)_2$ (Table 3).

Discussion

Bacillus circulans No. 4.1 produced chitinase continuously during the exponential phase of growth when grown in medium containing 0.3% colloidal chitin as substrate. The highest level of chitinase was found on the fifth day of culture when the bacterium entered the late stationary phase, then it gradually declined. This result may be due to the accumulation of chitinase in the medium during the previous growth phase.

A chitinase was purified from the extracellular culture of *B. circulans* No.4.1 by protein precipitation with 80% ammonium sulfate, a procedure similar to that used for *Aeromonas* sp. No.10S-24 [21] and *Trichoderma harzianum* [2]. Anion exchange DEAE-Sephacel chromatography, followed by gel filtration on Sephadex G-100 chromatography, produced a purified chitinase, shown to be a single band on SDS-PAGE. The molecular weight of the chitinase was 45 kDa, similar to that of chitinase reported for *Streptomyces* sp. S-84 [22] and *S. olivaceoviridis* [16].

The optimum pH of the *B. circulans* No. 4.1 chitinase was pH 8.0. A similar result has been obtained for *Alteromonas* sp. strain O-7 [20]. The optimum temperature of the chitinase was 40°C. Most chitinases reported from microorganisms had acidic p*I*s [24, 27] or alkaline p*I*s [14, 21], and the p*I* of chitinase of *B. circulans* No.4.1 was 5.1, similar to chitinase from *B. circulans* WL-12 [24]. Interestingly, this chitinase could hydrolyze colloidal chitin at a wide range of pH, including alkaline pH. This property should be very useful for including the chitinase as an enzyme supplement to a *B. thuringiensis*-based biopesticide to control lepidopteran larvae because the larval midgut has an alkaline pH [1].

The amino acid residues from the N-terminus of chitinase from *B. circulans* No.4.1 include several differences when compared with chitinases from *S. marcescens* QMB 1466 [11], *Alteromonas* sp. strain O-7 [20], *Bacillus* sp. strain MH-1 [17], and *B. circulans* WL-12 [23] (Table 4). This result agrees with other studies by Ohishi et al. [14] and Sakai et al. [17]. Moreover, Henrissat determined the amino acid sequences of various chitinases by hydrophobic cluster analysis and found that there was weak homology of those sequences [9].

The chitinase of *B. circulans* No.4.1 could hydrolyze both particulate chitin, i.e., colloidal chitin, purified chitin, and soluble chitin, i.e., glycol chitin, CM-chitin, and 4-MU-$(GlcNAc)_2$. This suggests that its chitin-binding domain possesses a strong binding capability for particulate chitin, which is similar to chitinases from *B. licheniformis* [19], *B. circulans* WL-12 [25], *Bacillus* strain MH-1 [17], and *S. olivaceoviridis* [16].

The results of this study indicate that the chitinase of *B. circulans* No.4.1 should be useful in the development of a new generation of *B. thuringiensis* on the basis of biopesticides based on genetic engineering technology.

ACKNOWLEDGMENT

This research work was supported by the National Center for Genetic Engineering and Biotechnology, the National Science and Technology Board, Thailand. We thank Dr. J. Marsh for critical reading of this manuscript.

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CURRENT MICROBIOLOGY Vol. 44 (2002), pp. 167–172
DOI: 10.1007/s00284-001-0084-7

Current Microbiology
An International Journal

Cloning, Sequencing, and Expression of a Chitinase-Encoding Gene from *Bacillus circulans* No. 4.1

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Received: 14 May 2001/Accepted: 22 June 2001

Abstract. A chitinase encoding gene from *Bacillus circulans* No. 4.1 was cloned in *Escherichia coli* by using pBluescript II SK. The recombinant plasmid containing the 2.6-kb chitinase gene was designated as pCHIB43. The nucleotide sequence revealed a single open reading frame containing 1794 bp and encoding 598 amino acids with a molecular mass of 65.78 kDa. The gene was sequentially deleted; the deletion clones were designated as pC66, pC6S, pSS6, and pEVS. The clones pC6S, pSS6, and pEVS hydrolyzed soluble chitin, but the ability to hydrolyze colloidal chitin was lost. The deduced amino acid sequence was investigated and found to be a chitin-binding domain and a catalytic domain containing 40 and 57 amino acid residues, respectively. A *Hin*dIII-*Sac*I fragment of pSS6 was subcloned into pBluescript SK to reverse the orientation of the gene, and the resulting plasmid pSK43 did produce chitinase. Thus, the cloned gene was expressed under the control of a self-promoter.

Chitinases are commonly found in a wide range of organisms, including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates [1, 5, 9]. The roles of these chitinases vary depending on their source. A major role in fungi, crustaceans, and insects is modification of their structural constituent chitin. By contrast, bacteria produce chitinase to digest chitin, primarily to utilize it as a carbon and energy source [7, 11, 15]. The chitinases are classified as endochitinase, exochitinase (EC.3.2.1.14), β-*N*-acetylglucosaminidase, and chitobiase (EC.3.2.1.30). Endochitinase splits the chitin polymer internally, exochitinase releases chitobiose from one end, β-*N*-acetylglucosaminidase releases *N*-acetyl-D-glucosamine (NAG) monomers, and chitobiase hydrolyzes chitobiose to NAG [2, 5]. Enzymatic hydrolysis of chitin to its constituent monomer is performed by a binary chitinase system [6]. The best known bacterial chitinase producers are in the genera *Aeromonas, Serratia, Myxobacter, Pseudoalteromonas, Vibrio, Streptomyces*, and *Bacillus* [4, 14, 24]. The distribution of chitinase and chitobiase in strains of *Bacillus* has been studied in detail, especially for the thermostable chitinase of *Bacillus licheniformis* X-70 [4, 19] and six distinct chitinase of *B. circulans* WL-12 [25].

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Our previous study demonstrated that *B. circulans* No. 4.1 could degrade colloidal chitin over a wide range of pH (pHs 6–12) and that a partially purified chitinase from this organism could enhance the toxicity of *Bacillus thuringiensis* subsp. *kurstaki* towards diamondback moth larvae [27]. Thus, we reasoned that the toxicity of *B. thuringiensis* toward target insects might be increased by the introduction of a chitinase-encoding gene.

The objective of this study was to clone the chitinase-encoding gene from *Bacillus circulans* No. 4.1 to express it in *Escherichia coli* and to analyze its nucleotide sequence. The ultimate goal was to transfer the cloned gene to *B. thuringiensis* in an attempt to improve its efficacy in the biological control of insect pests.

Materials and Methods

Bacterial strains and plasmids. *B. circulans* No. 4.1, a high chitinase producer, was isolated from soil in Thailand. It produced a large clear zone around colonies growing on nutrient agar supplemented with 0.3% colloidal chitin at 37°C [27]. The stock culture was maintained in 15% glycerol at −80°C. *Escherichia coli* DH5α was used as the cloning host. The plasmids pBluescript II KS and pBluescript II SK (Stratagene, USA) were used as cloning vectors.

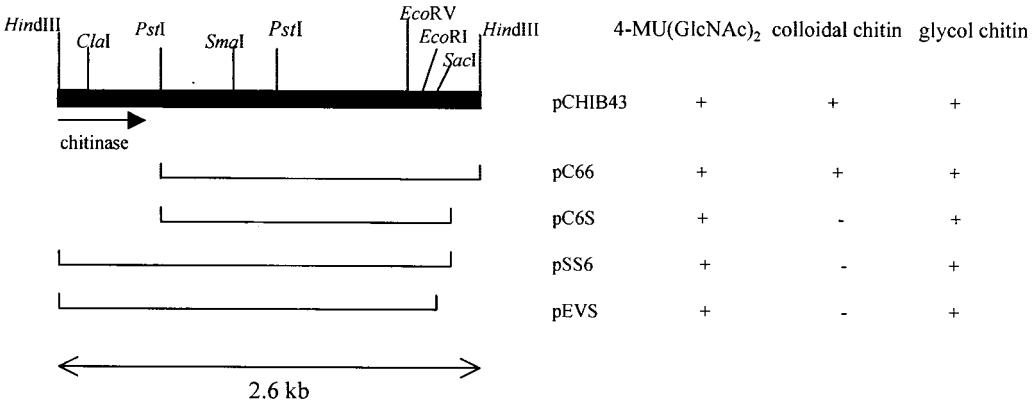

Fig. 1. Restriction map of the chitinase gene from *B. circulans* No. 4.1 and its deletion clones. Deletion clone derivatives were introduced into *E. coli* DH5α by transformation. The sizes of deletion fragments were estimated by agarose gel electrophoresis. The black box indicates the position of the open reading frame as determined by nucleotide sequencing. The arrow indicates the direction of transcription. The chitinolytic phenotype of each deletion clone is indicated by +, positive activity; −, negative activity.

Determination of chitinolytic activity.

Rapid method. Chitinolytic activity of the positive *E. coli* DH5α clones was determined by the rapid method of Tronsmo and Harman [22]. Bacterial colonies were grown on Luria-Bertani (LB) agar at 37°C overnight. Then the colonies were lysed with chloroform vapor for 15 min before overlaying with 5 ml of 0.7% Bacto agar containing 20 μg/ml of 4-methylumbelliferyl-β-D-*N*, *N*′-diacetylchitobioside [4-MU(GlcNAc)$_2$] (Sigma-Aldrich, USA) and incubation at 37°C for 15 min. A light blue fluorescence around colonies, as observed by UV transilluminator, indicated chitinolytic activity.

Detection of chitinase after separation by SDS-PAGE. E. coli clones harboring the chitinase gene were cultured in LB broth at 37°C overnight. Cells were then harvested and mixed with 2× sample buffer [10] and boiled for 5 min before samples (10 μl) were loaded onto an acrylamide gel (4% stacking gel, 12% separating gel). Electrophoresis was performed at 100 volts for 90 min in a discontinuous buffer system [10], after which gels were stained with Coomassie blue for 90 min and destained until protein bands were seen. Parallel gels were soaked in a large volume of refolding buffer [23] at 4°C for 16 h before overlaying with 0.7% Bacto agar containing 20 μg/ml of 4-MU(GlcNAc)$_2$ for fluorescence detection of chitinase activity by UV transilluminator.

Detection of chitinase activity in colloidal chitin agar. Positive chitinase clones were also confirmed by using colloidal chitin as the substrate. *E. coli* suspected of carrying the cloned chitinase gene were spotted on LB agar plates containing 100 μg/ml of ampicillin and 0.3% (wt/vol) colloidal chitin (colloidal chitin agar) prepared from crab shell chitin (Sigma-Aldrich, USA) as previously described [27], and were incubated at 37°C for 7 days in a sealed petri dish. Chitinolytic activity was indicated by the ability of bacterial colonies to produce hydrolytic clear zones.

Detection of chitinase in glycol chitin agar. Crude protein extracts from *E. coli* clones containing the chitinase gene were added to wells in Bacto agar plates supplemented with 0.1% glycol chitin prepared from purified chitin (Sigma-Aldrich, USA), as described by Trudel and Asselen [23], incubated at 37°C overnight. The plates were subsequently overlaid with 0.01% calcofluor white (Sigma-Aldrich, USA) and observed by UV transilluminator for evidence of enzyme activity. The light blue fluorescence area around wells was visualized.

Determination of chitinase activity by the colorimetric method. Chitinase activity was measured by the colorimetric method according to Ueda and Arai [24] by using 0.3% colloidal chitin and glycol chitin as substrates. Activity was calculated with a standard curve obtained from known concentrations of NAG (Sigma-Aldrich, USA) (0–0.15 mg). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of NAG per min under these conditions. Negative control tubes contained all components except substrate, and blanks contained all components except enzyme.

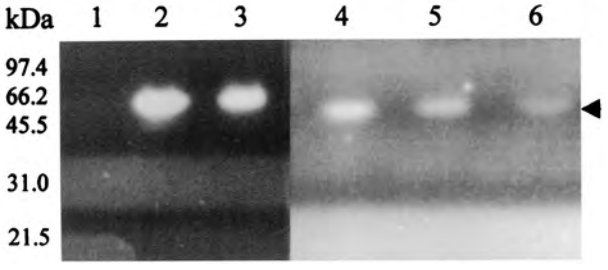

Fig. 2. Detection of chitinolytic activity of intracellular protein lysates from *E. coli* harboring different clones, after separation by SDS-PAGE in 12% separating gel. 4-MU(GlcNAc)$_2$ was used as the substrate. The arrowhead indicates the 65 kDa for chitinolytic proteins. Lane 1, protein molecular weight marker; lane 2, pCHIB43; lane 3, pC66; lane 4, pC6S; lane 5, pSS6; lane 6, pEVS;

Molecular cloning of the chitinase gene from *B. circulans* No. 4.1. A genomic library of *B. circulans* No. 4.1 was constructed beginning with a single colony of *B. circulans* No. 4.1 inoculated into 5 ml of LB broth, incubated at 37°C for 24 h on a rotary shaker (200 rpm). Cells were harvested, and DNA was prepared with a Genomic DNA preparation kit (QIAGEN, Germany). The DNA preparation was partially digested with *Hin*dIII and electrophoresed on 0.7% agarose. DNA fragments, 3–5 kb in size, were cut from the gel and extracted with a QIAGEN extraction kit before ligation with pBluescript II KS and transformation into *E. coli* DH5α by the heat shock method [16]. The transformed cells were spread on LB agar containing 100 μg ampicillin previously spread with X-gal (400 μg). Ampicillin-resistant white colonies were replicated on LB agar supplemented with 100 μg/ml of ampicillin and with or without colloidal chitin. The *E. coli* transformants growing on LB agar were screened by the rapid method with

4-MU$(GlcNAc)_2$ used as a substrate. Transformants that produced light blue fluorescence around colonies were confirmed for clear zones on colloidal chitin agar before recombinant plasmids were isolated from selected positive clones.

DNA sequencing and sequence analysis. Overlapping deletions of a 2.6-kb chitinase gene in plasmid pCHIB43 were obtained with a nested deletion kit purchased from Promega Corp. (USA). Fragments of appropriate deletion clones were sequenced using M13 universal and reverse primers with a dye terminator cycle sequencing kit (Perkin Elmer, USA) following the manufacturer's instructions. The sequencing was performed by an automatic DNA sequencer (ABI 373A, Applied Biosystem Inc., USA).

Nucleotide sequences of various clones were aligned and analyzed by using the DNASIS program (Hitachi Corp.). The promoter, SD sequence, initiation codon, terminator, and open reading frame (ORF) for the translated protein were determined in the complete nucleotide sequence.

Subcloning of the chitinase gene. Plasmid pCHIB43 was subjected to deletion on both ends, and deletion plasmids were subcloned as shown in Fig. 1. For construction of pC66, pCHIB43 was digested with *Cla*I and *Kpn*I. Linearized DNA fragments were digested with exonuclease III, followed with S1 nuclease. They were subsequently religated with T4 DNA ligase.

For construction of pSS6, pCHIB43 was digested with *Sac*I and religated to produce a transformant with 260 nucleotides deleted. For pEVS, pCHIB43 was digested with *Eco*RV and *Sac*I before linearized DNA fragments were further digested with S1 nuclease and religated with T4 DNA ligase to produce a transformant with 350 bp deleted. Finally, clone pC6S was constructed by cutting pC66 with *Sac*I, followed by religation.

All deletion derivatives were introduced into *E. coli* DH5α, and transformants were selected by overnight incubation at 37°C on LB agar supplemented with ampicillin (100 μg/ml). DNA inserts were sequenced as previously described.

Construction of recombinant plasmid pSK43. In order to test for the presence of a self-promoter in the cloned chitinase gene from *B. circulans* No. 4.1, a *Hin*dIII-*Sac*I fragment from pSS6 was digested and separated on 0.7% agarose gel. A 2.3-kb DNA fragment was cut from the gel, extracted with a QIAGEN extraction kit, and ligated with pBluescriptII SK at *Hin*dIII and *Sac*I sites to obtain a recombinant plasmid of opposite orientation. The ligated product was transformed into *E. coli* DH5α, and transformants harboring the recombinant plasmid were designated as pSK43.

Chitinase expression. *E. coli* transformants were cultured in 5 ml of LB broth containing 100 μg/ml ampicillin, at 37°C overnight. An aliquot of the overnight culture was inoculated at 1% into 250-ml Erlenmeyer flasks containing 50 ml of LB broth supplemented with ampicillin and further incubated at 37°C on a rotary shaker at 200 rpm for 6 h. The growth of each clone was determined by measurement of absorption at 600 nm. Culture broths were then centrifuged at 9,000 *g* for 10 min in a refrigerated centrifuge to remove cells. Cells were washed with distilled water and resuspended in 1 ml of the same for sonication (Soniprep 150, Sanyo) at a 60 sec on and 30 sec-off cycle until the suspension became clear. Then, the lysate was centrifuged at 9,000 *g*, 4°C, for 30 min (Kontron). The supernatant was then sterilized by filtration through a 0.45-μm membrane filter before storage at −20°C until used.

Protein determination. The protein concentration of enzyme preparations was determined following the method described by Bradford [3], with bovine serum albumin as the standard (0.2–1 mg/ml).

Results

Cloning of the chitinase gene. Two clones from the genomic DNA library of *B. circulans* No. 4.1 were found to be positive for chitinase activity with both the 4-MU$(GlcNAc)_2$ and colloidal chitin agar assays. Both clones contained the same size of chitinase gene insert (2.6 kb). One plasmid was selected and designated as pCHIB43. Its restriction enzyme map is shown in Fig. 1.

Nucleotide sequence analysis of the chitinase gene. Plasmid pCHIB43 was prepared for sequencing by generation of 12 nested sets of deletion clones containing various sizes of DNA fragments. Nucleotide sequences were joined and aligned. The complete nucleotide sequence of the 2580-bp chitinase gene from *B. circulans* No. 4.1 was obtained (GenBank AF154827). G+C residues and A+T residues of the coding sequence accounted for 45.57 mol% and 56.37 mol%, respectively. A single ORF containing 1794 bp extended from the ATG start codon at position 727 and ended at the TAA stop codon at nucleotide 2521. The deduced amino acid sequence of the chitinase gene contained 598 amino acid residues with a calculated molecular mass of 65.78 kDa. A region upstream of the putative ATG start codon contained the sequence TGCAGC at nucleotides 620 to 625, similar to the −35 consensus sequence, and the sequence TATAAA was found at nucleotides 647 to 652, similar to the −10 consensus sequence. A putative SD sequence, GAAAA, was found at nucleotides 718–722 with a 5-bp spacer upstream from the ATG start codon. Downstream of the termination codon TAA, a putative inverted repeat sequence was found at nucleotides 2556–2580 and was composed of a 9-bp stem and a loop of six bases. This putative hairpin-loop structure probably represented a transcriptional termination signal.

The deduced amino acid sequence of chitinase was investigated and found to be a catalytic domain (Fig. 3) and chitin-binding domain (CBD) (Fig. 4) containing 57 (residues 148–204) and 40 (residues 549–588) amino acid residues, respectively.

Deletion analysis and subcloning of the chitinase gene. Plasmid pCHIB43 was subjected to deletion on both ends as shown in Fig. 1. The four deletion clones designated as pC66, pSS6, pEVS, and pC6S were examined for chitinolytic activity by plate assay by using 4-MU$(GlcNAc)_2$, colloidal chitin, and glycol chitin as substrates. All hydrolyzed the soluble chitins, glycol chitin, and 4-MU$(GlcNAc)_2$, but pCHIB43 and pC66 hydrolyzed insoluble chitin, colloidal chitin.

Chitinase gene self promoter. Four clones containing recombinant plasmids with the chitinase gene in opposite orientation were obtained, and the clones containing

```
                                                                          *
                            ↓  ↓                                    ↓   ↓
B.circulans 4.1     148 PHLKTFISVGGWTWSNRFSDVAADPVARGN-FAASAVEFLRKYGFDGVDLDWEYPVSG 204
A.album             121  NMKVMLSIGGWTWSTNFPAAASSAATRKT-FAQSAVGFMKDWGFDGIDIDWEYP    173
B.subtilis          147 PHLKTFISVGGWSWSNRFSDVAADPAARE--FAASAVNFLRKYGFDGVDLDWEYPVSG 203
B.circulans chiA    153 PHLKTFISVGGWSWSNRFSDVAADPAAREN-FAASAVNFLRKYGFDGVDLDWEYPVSG 209
B.thuringiensis     158 PHLKTIISVGGWTWSNRFSDMAADEKTRKV-FAESTVAFLPAYGFDGVDLDWEYP    211
C.tentans            93  HLKVLLAIGGWNEGSRNYSDMAGDPTRRGRFVKQTVSFIKQHNFDGLDLDWEYP    146
C.thermocellum      112 PHVKTLISVGGWTESKYFSDVALTEESRNT-FADSCVEFIRTYRFDGVDIDWEYPVSG 168
K.zopfii            153 PNLKTIISVGGWTWSNRFSDVAATAATREV-FANSAVDFLRKYNFDGVDLDWEYPVSG 209
M.anisopliae        122  NMKTMLSIGGWTWSTNFPAAASTAATRSN-FAKSAVTIMKDWGFDGIDVDWEYP    174
M.flavoviride       123   MKTMLSIGGWTWSTNFPAAASTAATRSN-FAKSAVTIMKDWGFDGIDVDWEYP    174
T.hamatum U88560    122  NLKVMLSIGGWTWSTNFPSAASTDANRKN-FAKTAITFMKDWGFDGIDVDWEYP    174
T.harzianum U49455  123   LKVLLSIGGWTWSTNFPSAASTDANRKN-FAKTAITFMKDWGFDGIDIDWEYP    174
T.harzianum S78423  122   LKVLLSIGGWTWSTNFPSAASTDANRKN-FAKTAITFMKDWGFDGIDIDWEYP    173
T.harzianum L14614  122  NLKVMLSIGGWTWSTNFPSAASTDANRKN-FAKTAITFMKDWGFDGIDVDWEYP    174
T.hamatum Z71415    122  NLKVMLSIGGWTWSTNFPSAASIDANRKN-FAKTAITFMKDWGFDGIDVDWEYP    174
T.virens            123   LKVLVSIGGWTWSTNFPSAASTDANRKN-FARTAITFMKDWGFDGIDVDWEYP    174
      Consensus             K   S GG                    F  S      @ FDG D D E
```

Fig. 3. Comparison of putative catalytic regions in chitinases. Conserved residues are shown in bold print. Arrows indicate the four residues identified as being conserved among bacterial and fungal chitinases. Asterisk (*) indicates residue necessary for activity, and conserved aromatic residues are underlined. The putative catalytic domain of *B. circulans* No. 4.1 was compared with the catalytic domains of *Aphanocladium album* X64104, *B. subtilis* AF069131, *B. circulans* A1 M57601, *B. thuringiensis* U89796, *Chironomus tentans* Y13234, *Clostridium thermocellum* Z68924, *Kurthia zopfii* D63702, *Metarhizium anisopliae* AF027498, *M. flavoviride* AJ243014, *Trichoderma hamatum* U88560, *T. hamatum* Z71415, *T. harzianum* L14614, *T. harzianum* S78423, *T. harzianum* U49455, and *T. virens* AF050098.

```
B.circulans No. 4.1      549 YDEWKE-TNAYTGGERVAFNGKVYE---AKWWTKGDRPDQS--GEW  588
B.circulans A1           653 VSAWQVNTA-YTAGQLVTYNGKTYKCLQ---------PHTSLAG-W  688
B.circulans D             31 AAQWQAGTA-YKQGDLVTYLNKDYECIQPH--TAL----T---G-W   66
B.subtilis               547 YNEWKD-TAVYTGGDRVVFNGKVYE---AKWWTKGEQPDQA--GE   585
Aeromonas 10S-24         395  --WSSSTA-YNGGATVAYNGHNYQ---AKWWTQGNVPSSS       428
Alteromonas chiA         776 YPTWDRSTV-YVGGDRVIHNSNVFE---AKWWTQGEEPGTADV--W  815
Pseudoalteromonas chiB   439  --WDASKA-YNAGDKVSYLGTVYK---AKWWVQGEKPNSSDA--W  475
Pseudoalteromonas ChiC   782 ---WNADAI-YTGGDTVLYNGVEYK---AKWWTQGDRPDL---GPW  818
S.griseus                 36  --WSSSSV-YTNGGTVSYNGRNYT---AKWWTQNERPGTSDV--W   72
       Concencus                W   T  Y  G  VS  G  @     A W@T G  P T   G W
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Fig. 4. The putative chitin-binding domain (CBD) of *B. circulans* No. 4.1 compared with the putative chitin-binding domains of *B. subtilis* AF069131, *B. circulans* A1 M57601, *B. circulans chiD* D10594, *Pseudoalteromonas* sp. *chiC* AF007896 [21], *Alteromonas* sp. *chiA* D13762, *Pseudoalteromonas* sp. *chiB, Streptomyces griseus chiC* AB009289 [12], *Aeromonas* sp. 10S-24 D31818. Conserved residues are in bold print, and closely related amino acids are underlined. Symbol @ indicates conserved aromatic residue.

recombinant plasmid pSK43 with a 2.3-kb *Hin*dIII-*Sac*I insert gave positive chitinase activity detecting with 4-MU(GlcNAc)$_2$ and glycol chitin as substrates. It was concluded that the chitinase gene was expressed by its own promoter. However, when this transformant was cultured on 0.3% colloidal chitin agar, it gave no clear zone around colonies within 1 week. The result was the same as for cloned, containing plasmid pSS6 with a 260-bp deletion at the right terminus of pCHIB43 (Fig. 1).

Molecular mass. The chitinases produced by *E. coli* harboring pCHIB43, pC6S, pSS6, pEVS, and pC66 were separated by SDS-PAGE, after which gels were soaked in refolding buffer and overlaid with 4-MU(GlcNAc)$_2$ for detection of chitinase activity bands by UV transilluminator. Results showed that the molecular mass of the chitinase was approximately 65 kDa, but the chitinase produced by deletion clones containing pC6S, pSS6, and pEVS was smaller than the original one (Fig. 2).

Expression of the chitinase gene in *E. coli*. Chitinase produced by clones containing the plasmids pCHIB43 pSK43, pC66, pC6S, pSS6, and pEVS was quantitated by the colorimetric method with both glycol chitin and colloidal chitin as substrates. Lysates were prepared from equal cell numbers, and the protein concentration of cell lysates was used to calculate specific activities of chitinase for each clone. Specific activities for *E. coli* clones harboring pCHIB43, pC66, pC6S, pSS6, and pEVS were

60.8 mU/mg, 22.53 mU/mg, 36.11 mU/mg, 5.03 mU/mg, and 0.96 mU/mg, respectivly. In conclusion, the clone harboring pCHIB43 had the highest activity, while clones harboring pC66, pC6S, pSS6, or pEVS had lower activity.

Chitinase characteristics. *E. coli* containing pCHIB43 from *B. circulans* No 4.1 was tested for chitinase specificity with 4-MU$(GlcNAc)_2$, colloidal chitin, and glycol chitin. The best substrate was 4-MU $(GlcNAc)_2$ when compared with colloidal chitin and glycol chitin. With substrate analogs such as 4-methylumbelliferyl 4-MU$(GlcNAc)_{1–3}$, light blue fluorescence was obtained from 4-MU$(GlcNAc)_2$ and 4-MU$(GlcNAc)_3$, but not from 4-MU$(GlcNAc)_1$. Such activity is characteristic of endochitinase.

Discussion

Nucleotide sequence and deduced amino acid sequence. The cloned gene encoded a putative chitinase enzyme of 598 amino acids with a calculated molecular mass of 65.78 kDa. This corresponded well with 65 kDa as determined by SDS-PAGE analysis of the chitinase produced by *E. coli* clones containing pCHIB43 and its derivatives. The gene sequence contained all the elements necessary for transcription, including a self-promoter region located upstream of the ORF.

When the complete nucleotide sequence was compared with known bacterial chitinase genes from the Genbank database, similarity was observed among chitinase genes of Gram-positive bacteria. The nucleotide sequence of the chitinase gene from *B. circulans* No. 4.1 has 86% similarity to that of *B. licheniformis* [20], 64% similarity to that of *B. subtilis* (AF069131), 56% similarity to that of *Kurthia zopfii* (D63702), 54% similarity to that of *B. circulans* A1 (J05599), and 51% similarity to that of *B. thuringiensis* (U69796). The putative amino acid sequence from *B. circulans* No. 4.1 showed 87% homology with chitinase from *B. licheniformis* and 84% with chitinase from *B. subtilis*.

Catalytic domain. The putative catalytic domain of 57 amino acids was compared with other catalytic domains of chitinase (Fig. 3). Some residues appeared to be conserved among the bacterial chitinases [18], although the overall similarity was low. We determined and found a consensus catalytic domain of *B. circulans* No. 4.1 chitinase consisting of 13 residues, with Asp (195) and Glu (199) being the most conserved residues. Watanabe and co-workers also found that Asp-200 and Glu-204 were the residues directly involved in the catalytic mechanism of chitinase A1, through a manner similar to Glu-35 and Asp-52 residues of lysozyme [26]. It was reported that *Alteromonas sp.* 0-7 employed Asp-290 and Glu-292 residues for chitin cleavage by β-chitinase [8].

Chitin-binding domain (CBD). The conservation of CBD in other chitinases and in related enzymes was examined. It consisted of 15 or 17 residues, including conserved aromatic residues (Fig. 4). The aromatic amino acids appear to be essential for hydrophobic binding of 1,4-β-glycanases to polysaccharides [13]. It was found that the CBD of *B. circulans* No. 4.1 (Fig. 4) was similar to the CBDs of various bacterial chitinases as shown in Fig. 4.

Expression of *B. circulans* No. 4.1 chitinase gene in *E. coli*. The chitinase gene from *B. circulans* No. 4.1 produced a protein with a molecular mass of 65 kDa. Trimming of the 3′ end of the gene in pCHIB43 led to decreased activity. The lower activity of clones harboring the truncated gene appeared to be due to removal of the 68 amino acids in pSS6 and 99 amino acids in pEVS at the carboxy terminal region of the protein. By contrast, deletion of 566 nucleotides from a *Hin*dIII site at the 5′ end of pC66 did not affect activity for degradation of colloidal chitin, although activity was lower than with pCHIB43, indicating that the region removed was necessary for full activity of the enzyme. This deleted upstream, non-coding part of the ORF contained the ribosome-binding site.

Clones containing pSS6, pC6S, and pEVS were missing the CBD located at the 3′ end of the gene, and this probably resulted in decreased effectiveness of the enzyme. It suggested that the C-terminal region was important for strong affinity of the enzyme to insoluble chitin [25]. In contrast, deletion of the CBD from the *B. circulans* chitinase A1 reduced the rate of hydrolysis of colloidal chitin by approximately 50% [26]. Svitil and Kirchman [18] reported that *ChiA* without the CBD did not bind to chitin, but could hydrolyze glycol chitin [17].

ACKNOWLEDGMENTS

This research work was supported by the National Center for Genetic Engineering and Biotechnology, the National Science and Technology Development Agency, Thailand. We thank Prof. Timothy Flegel for critical reading of this manuscript.

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Am. J. Trop. Med. Hyg., 69(2), 2003, pp. 206–212

ENHANCEMENT OF THE EFFICACY OF A COMBINATION OF *MESOCYCLOPS ASPERICORNIS* AND *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* BY COMMUNITY-BASED PRODUCTS IN CONTROLLING *AEDES AEGYPTI* LARVAE IN THAILAND

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Abstract. Prolonged efficacy of a combination of bacteria (*Bacillus thuringiensis* var. *israelensis* [Bti] and copepods (*Mesocyclops aspericornis*) in controlling immature forms of *Aedes aegypti* in peridomestic water containers was achieved by adding various products from local villages as supplementary food for copepods. In all experiments, 100 first-instar larvae were added into the breeding containers every day for eight weeks. Combinations of biological control agents and each local supplementary food were applied once at the beginning of the experiment. At the end of the experiment, the average number of mosquito larvae in containers with a combination of copepods and Bti with one gram of rice grain had decreased to only 0.5% of that with no control agent. In comparison, the average numbers of mosquito larvae in containers with Bti only, or copepods only, were approximately 10% and 33% of those in containers with no control agents, respectively. In addition, the number of copepods in containers with mosquito larvae and supplementary food was at least three times higher than those with mosquito larvae alone.

INTRODUCTION

Dengue fever is a serious infectious disease in several tropical and subtropical countries in Asia, Africa, and The Americas.[1] It causes more illness and death than any other arboviral infection, and no commercial vaccine for dengue is currently available.[2] Dengue fever is caused by an RNA flavivirus. The spread of dengue throughout the world is mainly attributed to proliferation and adaptation of its mosquito vectors.[1] Once infected by the virus, the mosquito vectors remain viruliferous for the rest of their life. The main vector of dengue flavivirus is *Aedes aegypti* L., which is anthropophilic and common in urban and suburban environments.[2] In nature, *Ae. aegypti* relies mainly on rainfall for breeding. However, it has adapted to urban and suburban environments as well, where it uses human-made peridomestic containers such as clay jars and vases for breeding regardless of the rains.[3] Therefore, attempts to control dengue have been aimed mostly at controlling the *Ae. aegypti* mosquito vectors.[4]

A variety of methods have been used to control *Aedes* mosquitoes. Application of oil and sulfur fumigation, larvicide fogging, and other insecticides work well in the short-term, but after a few weeks mosquito populations inevitably increase and eventually develop resistance against these chemicals.[5] Biocontrol efforts have included the use of predatory fish,[6] plankton management, and the application of copepods[7,8] and *Bacillus* spp.[9,10] Cultural methods, such as removal of breeding sites, are sometimes effective, depending on the scale of urbanization.[11] Integration of these methods could provide a more cost-effective and environmentally friendly approach in controlling control mosquito vectors.[12,13]

Copepods are small aquatic crustaceans. Observations of copepod predation on first-instar mosquito larvae[14] led to the first investigation in 1981[15] of their potential as biological control agents. Since then, various species of predacious copepods have been tested for their potential to control mosquitoes. Most of them are omnivorous and prey on immature mosquitoes, especially first-instar larvae, but rarely on later stages.[16,17] Several species of copepods, including *Mesocyclops aspericornis, M. thermocyclopoides, M. guangxiensis,* and *M. longisetus*, have been reported as potential biological control agents of *Ae. aegypti*.[7,18–20] *Mesocyclops aspericornis* is a species known for its controlling efficacy against several species of mosquito larvae and for its wide distribution. Surveys have shown that *M. aspericornis* is abundant in natural freshwater sources[21–23] (Vihokto S, 1994. Preliminary survey and experimental studies of *Mesocyclops* spp. as biological control agents of dengue vectors in a rural Thai community. M.S. Thesis, Bangkok, Thailand: Mahidol University). *Mesocyclops aspericornis* is a dominant species found in artesian wells and peridomestic containers in eastern Thailand (Vihokto S, 1994. Preliminary survey and experimental studies of *Mesocyclops* spp. as biological control agents of dengue vectors in a rural Thai community. M.S. Thesis, Bangkok, Thailand: Mahidol University). It has been shown to be an effective predator of *Ae. aegypti* larvae in both laboratory experiments and field trials.[23,24]

Entomopathogenic bacteria have been alternatives to chemical insecticides for decades. Several soil bacteria have been isolated and characterized, including *Bacillus thuringiensis* var. *israelensis* (Bti). Proteinaceous endotoxins in the crystalline spores of Bti are lethal to mosquito larvae upon ingestion.[25] A number of formulations have been developed and tested against *Ae. aegypti* and many other mosquito larvae.[26,27] The endotoxins produced by Bti are very specific to certain types of dipterans, including mosquitoes (Culicidae) and black flies (Simuliidae).[25,28] Application of Bti in a natural setting is relatively safe and ecologically acceptable to non-target organisms and has no detrimental effects on humans.[29,30] However, its limitation is the high sensitivity of *Bacillus* spp. to environmental factors.[9,27,31]

An effective control of mosquito larvae was attained when *M. aspericornis* and Bti were combined.[10] Nevertheless, reapplication of Bti may be necessary to achieve satisfactory control levels because the controlling efficacy of Bti decreases a few days after application under natural settings. In addition, effectiveness of using copepods to control mosquito larvae greatly depends on the availability of their food.[7]

To prolong the efficiency of a combination of *M. aspericornis* and Bti in controlling *Ae. aegypti*, we investigated the

potential of using products readily available from the local community as copepod supplementary food in peridomestic containers. The copepods prey mostly on the newborn mosquito larvae due to size limitation, whereas Bti could kill most mosquito larvae upon application. Addition of food for the copepods could then supplement their natural food source, sustain the copepod population, and enhance controlling efficacy of the combination to achieve a practical long-term control of *Ae. aegypti* vector.

There were two sets of experiments in this study. The first experiment was conducted to determine a suitable local product as copepod supplementary food. The second experiment was conducted to study the effects of supplementary food on the efficacy of copepods and Bti for controlling *Ae. aegypti* mosquito larvae.

MATERIALS AND METHODS

Test facilities. We used human-made 200-liter peridomestic containers for the experiment. These containers are the same kind that villagers in rural Thailand use for storing rainwater for their everyday use. The containers were maintained under ambient temperature within a greenhouse of the Faculty of Science, Mahidol University (Bangkok, Thailand). Each container was filled with 150 liters of dechlorinated water and covered with mosquito net to prevent any contamination from local mosquitoes. The first experiment was performed once in summer (July) with two replicates for each treatment. The second experiment was performed once in summer (July–August) and once in winter (December–January) with two replicates for each treatment. The mean ± SD water temperature during the experiment conducted in summer was 28.8°C ± 0.38°C. The mean ± SD water temperature during the experiment conducted in winter was 26.53°C ± 2.49°C.

Sampling methods. Copepods and living mosquito larvae were sampled from each container with a very fine round sweep net with a diameter of 25 cm. The net was swept gently 10 times in a spiral manner from the surface to the bottom of the water. Copepods and mosquito larvae were separated and counted. After counting, third-instar and fourth-instar mosquito larvae and pupae were discarded since they were too large for successful predation by the copepods, whereas the copepods and first-instar and second-instar mosquito larvae were re-inoculated into the same container from which they were taken. The net was then put in boiling water for five minutes and washed before each use to avoid cross contamination of the samples.

Liquid Bti preparation and application. Liquid Bti was produced at the Department of Biotechnology, Faculty of Science at Mahidol University. Bti IPS82 was used as a standard to titrate the Bti used in this experiment because it has been produced since 1982 and its potency is considered stable.[32] The International Toxic Unit (ITU) of Bti used in this study was 531 ITU/mg when compared with Bti IPS82 at 25 ± 2°C (mean ± SD) (Chansang U, Bhumiratana A, Kittayapong P, unpublished data). Its 50% and 90% lethal concentrations (LC_{50} and LC_{90}) were 2.3×10^{-4} and 5.3×10^{-4} ml/L, respectively (Chansang U, Bhumiratana A, Kittayapong P, unpublished data). A concentration 20 times that of the LC_{90} was used in this study.

Mass rearing of predatory copepods. Copepods were collected from their natural breeding sites at Ban Laem Hin, Hua Sam Rong Subdistrict, Plaeng Yao District, Chachoengsao Province in eastern Thailand. Isofemale lines were established from gravid females and maintained at the Department of Biology, Faculty of Science, Mahidol University (Bangkok, Thailand). More than 90% of the collected samples were identified and confirmed by Dr. Janet W. Reids (National Museum of Natural History, Smithsonian Institution, Washington, DC) to be *Mesocyclops aspericornis* (Daday) (Vihokto S, 1994. Preliminary survey and experimental studies of *Mesocyclops* spp. as biological control agents of dengue vectors in a rural Thai community. M.S. Thesis, Bangkok, Thailand: Mahidol University). Once identified, gravid female *M. aspericornis* from different isofemale lines were pooled and mass-reared in dechlorinated water (pH 7) in a 15-liter fish tank at 27 ± 2°C (mean ± SD). *Paramecium* spp. prepared from boiled rice straw water extract and commercial powdered fish food were used to support the copepod cultures. We sampled 100 copepods to apply as the initial number to each treatment that contained copepods as a control agent.

Mosquito larvae. First-instar larvae of *Ae. aegypti* were kindly provided by the Center for Vectors and Vector-Borne Diseases, Faculty of Science at Mahidol University. The colony was established in 1994 from field-collected larvae. The original location was from Hua Sam Rong Subdistrict, Plaeng Yao District, Chachoengsao Province. This colony has never been exposed to any insecticides or Bti products.

Test of suitable local supplementary food for copepods. This experiment was conducted in peridomestic containers similar to those that villagers in rural Thailand use to store rainwater for their everyday use. The water in these containers was generally kept clean, but contained a limited amount of organic materials. Limited organic sediments and mosquito larvae may not be sufficient to sustain a population of *M. aspericornis* used to control mosquito larvae in a long-term control strategy. Thus, products from the local community used as supplementary food for the copepods might help sustain the numbers of copepods when their natural food is scarce.

We tested low-cost local natural products that seemed of practical use for villagers. In this experiment, we used clean 200-liter peridomestic containers filled with 150 liters of dechlorinated water. The experiment was conducted by inoculating 100 adult *M. aspericornis* to every container. One hundred first-instar *Ae. aegypti* larvae were then supplied to each container daily as the regular copepod food. There were five treatments in this experiment and two replications for each treatment. In the control containers, there was no supplementary food. We used one and five grams of rice grain (*Oryza sativa* L. [Poaceae]); five grams of commercial fish food granules; or five grams of sesban leaves (*Sesbania grandiflora* L. [Fabaceae]) as the supplementary food for the copepods. All materials were sterilized before applying to the treatment containers. The supplementary food was supplied only once at the beginning of the experiment. Copepods were sampled and counted every seven days. This experiment was terminated at the end of the fourth week.

Copepods and Bti with supplementary food for controlling mosquito larvae. We tested the efficacies of Bti, copepods, and their combination with local products as copepod supplementary food for controlling *Ae. aegypti* larvae in peridomes-

tic containers. We divided containers into five treatments with two replications.

The experiment was conducted by inoculating 100 first-instar *Ae. aegypti* larvae into each container daily to simulate high numbers of *Ae. aegypti* in nature. The first treatment (L) was the control containers in which 0.1 grams of commercial fish food granules was added with mosquito larvae, but without Bti nor copepods. In the second treatment (L + C), we inoculated 100 adult *M. aspericornis* as the only control agent. In the third treatment (L + B), liquid Bti was used as the only control agent. In the fourth treatment (L + C + B + R), a combination of Bti and 100 adult copepods were used as control agents and one gram of rice was added as supplementary food for the copepods at the beginning and during the fourth week of the experiment. In the fifth treatment (L + C + R), we inoculated 100 adult *M. aspericornis* as the only control agent and one gram of rice was supplied at the beginning and during the fourth week of the experiment. The fifth treatment was performed only in the winter experiment. Living mosquito larvae and copepods were sampled and counted every seven days. This experiment was terminated at the end of the eighth week.

RESULTS

Experiment on supplementary food for copepods. The average number of copepods that survived in each treatment is shown in Figure 1. At the end of the fourth week when this experiment was terminated, the average number of copepods in the treatment with sesban leaf (367) was more than twice that of the control (168). The number of copepods in the treatment with one gram of rice (609) was more than three times that of the control (168). The numbers of copepods in the treatment with five grams of rice (1,154) or five grams of commercial fish food (1,214) were each more than six times that of the control (168). Nevertheless, the water appeared cloudy and polluted in the latter two treatments.

Supplementary food and controlling efficacy of copepod and Bti. The numbers of living mosquito larvae (M) and copepods (C) from each treatment of the summer and winter experiments are shown in Figure 2A and B, respectively. By the end of the experiment, the number of living mosquito larvae in the control, L (M), was at least twice as many as those of other treatments. The number of living mosquito larvae from the treatment that combined Bti and copepods with supplementary food, L + C + B + R (M), was the least throughout both summer and winter experiments. The treatment with Bti only (L + B) kept the number of living mosquito larvae very low, although the number of living mosquito larvae, L + B (M), increased in the fifth week of the winter experiment and in the seventh week of the summer experiment. These seemed to be signs of loss of larvicidal activity of Bti in the treatment containers. The numbers of living mosquito larvae in the treatments, in which Bti or copepods was the only control agent, L + B (M) and L + C (M), respectively, were not significantly different. However, there was a persistent contamination problem with ostracods in the treatment of mosquito larvae with copepods in the winter experiment that clearly affected the numbers of copepods, L + C (C), and living mosquito larvae, L + C (M). For example, there was an increase in the number of copepods in the fifth week of the summer experiment, but the number of copepods in the same treatment, L + C (C), in the winter experiment was at least five times less. The major decrease in the number of copepods could be attributed to the increases of living mosquito larvae and the lack of controlling agents. The number of living mosquito larvae, L + C (M), in the fifth week of the winter experiment was at least eight times higher than that of the summer experiment during the same period.

Effects of seasonal changes on the number of copepods

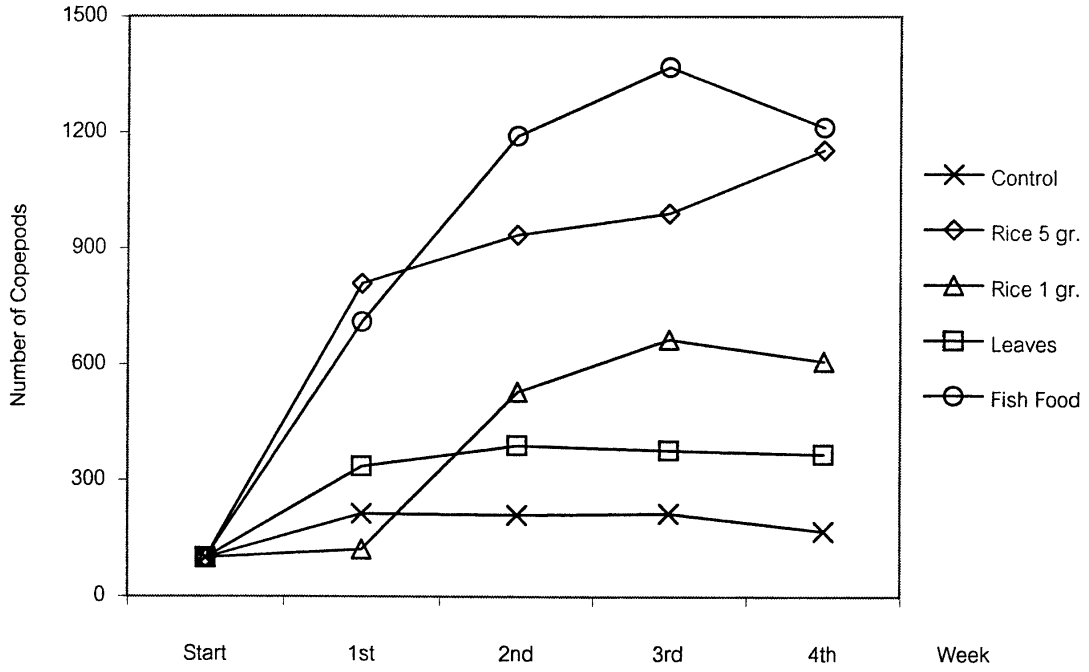

FIGURE 1. Average number of *Mesocyclops aspericornis* copepods in response to different supplementary foods. gr. = grams.

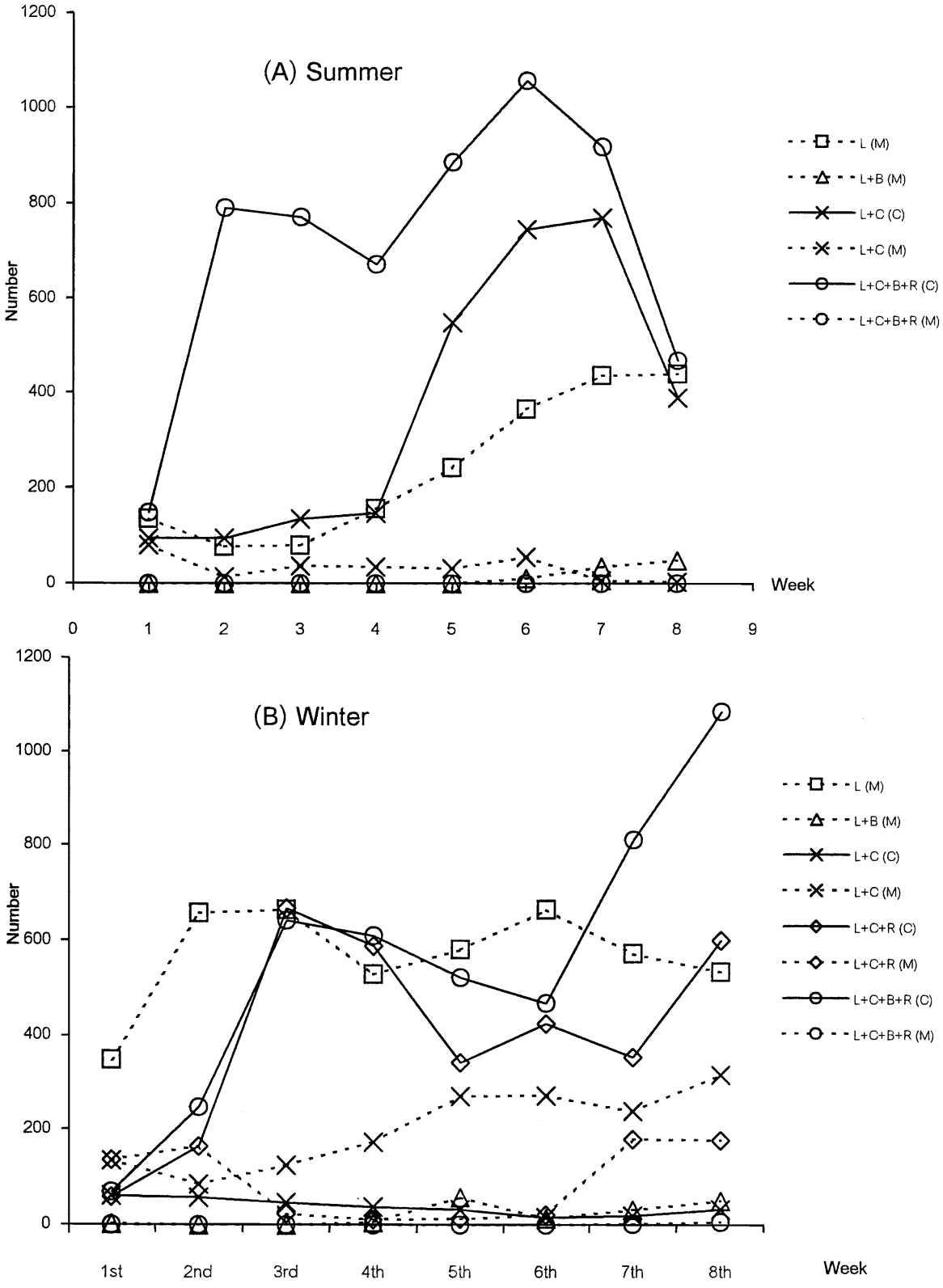

FIGURE 2. Average number of *Mesocyclops aspericornis* copepods (**solid lines** [**C**]) and living *Aedes aegypti* mosquito larvae (**dotted lines** [**M**]) from **A**, summer (July–August) and **B**, winter (December–January) experiments. L = *Ae. aegypti* larvae; L+B = *Ae. aegypti* larvae and *Bacillus thuringiensis* var. *israelensis* (Bti); L+C = *Ae. aegypti* larvae and *M. aspericornis*; L+C+R = *Ae. aegypti* larvae, *M. spericornis*, and one gram of rice; L+C+B+R = *Ae. aegypti*, *M. aspericornis*, Bti, and one gram of rice.

were observed, especially in the treatment of mosquito larvae, copepods, and Bti with supplementary food (L + C + B + R). Fluctuations in the numbers of copepods and living mosquito larvae were also observed (Figure 2). Supplementary food had noticeably positive effects on the numbers of copepods. The average number of copepods of the treatment provided with supplementary food, L + C + B + R (C), was generally greater than the average number of copepod with supplementary food, L + C + R (C), and at least twice that of the treatment provided with mosquito larvae alone, L + C (C). Conversely, the average number of living mosquito larvae in the treatment provided with supplementary food, L + C + B + R (M), was the least throughout the experiment when compared with that of other treatments: L + C + R (M), L + C (M), and L + B (M).

DISCUSSION

Mesocyclops aspericornis belongs to the lower food chains of complex aquatic ecosystems. In a complex system of a large body of water, this copepod may have little direct impact on mosquito larval populations. In restricted conditions, however, where the copepods become dominant, *M. aspericornis* could be both a predator and a competitor for food of mosquito larvae.[10,17] *Mesocyclops* spp. can effectively reduce the number of *Ae. aegypti* in both laboratory and natural settings.[10,23] In some instances, introduction of *M. aspericornis* in conjunction with other controlling methods resulted in the eradication of *Ae. aegypti*.[7,21,22,33,34] In this study, we investigated a combination of *M. aspericornis* and Bti in the long-term control of the anthropophilic *Ae. aegypti* mosquito in peridomestic containers by adding natural supplementary food available from the local community.

One of the important factors influencing the efficacy of using *M. aspericornis* to control mosquito larvae has been its ability to subsist in containers regularly used by people.[7,35] In rural Thailand, villagers use large peridomestic containers to store rainwater for their everyday use and keep the containers relatively clean, resulting in minimal contamination with organic materials. Application of *M. aspericornis* to these containers to effectively control the mosquito larvae may require the addition of supplementary food to sustain *M. aspericornis* populations for the long-term control of *Ae. aegypti*.

Based on the results of this study, a good candidate as copepod supplementary food without compromising the usability of the water was one gram of rice per 150 liters of water, although re-application was necessary as the supplementary food was depleted. Although the containers with commercial fish food granules and those with five grams of rice produced the very high numbers of copepods, the water became so polluted that it was not suitable for domestic use by villagers. There was little response of copepods to sesban leaves when compared with the control, in which no supplementary food was provided.

A combination of *M. aspericornis* and Bti for the long-term control of *Ae. aegypti* in rural Thailand has great potential. The copepods are very successful as predators of the first-instar mosquito larvae,[14,16,17] but are not effective predators of larger mosquito larvae. Thus, a combination of approaches may lead to more satisfactory control of mosquitoes. *Bacillus thuringiensis* var. *israelensis* has been used in conjunction with *M. aspericornis* because of its high toxicity and high specificity to mosquito larvae.[10,12] In addition, Bti shows no detrimental effects on either copepods or humans and has relatively minor effects on non-target organisms.[30,36,37]

We showed that the number of surviving mosquito larvae was lowest and the number of copepods was highest in the treatment containing copepod supplementary food. This implies that efficacy of the combination of copepods and Bti to control *Ae. aegypti* mosquito larvae in peridomestic containers could be prolonged and enhanced by addition of copepod supplementary food.

It is possible that seasonal differences between summer (July–August) and winter (December–January) experiments could influence copepod populations within the containers. Decreases in copepod numbers in the second week of the summer experiment and in the third week of the winter experiment may be due to depletion of food sources. In the summer experiment, copepods in the treatment with Bti and copepods with supplementary food may have consumed the supplementary food faster than they did in the winter experiment. In addition, the copepod populations from the winter experiment seemed to take a longer time to replenish, after addition of supplementary food during the fourth week of the experiment, than those in the summer experiment. It has been reported that the duration of *Ae. aegypti* larval development varies depending on the availability of food resources, but inversely with the temperature.[38] This could subsequently affect the population dynamics of the copepods, since the copepods responded to the availability of the supplementary food more rapidly in the summer experiments than in the winter ones.

The ostracods interfered with the ability of the copepods to control the mosquito larvae. Contamination with ostracods was observed in the containers with copepods as the sole control agent in the winter experiments. The ostracod contamination apparently decreased the number of copepods, since the ostracods may have competed for food with early larval stage of copepod or nauplii. As a result, it increased the number of surviving mosquito larvae. It was suggested that using leaves to cover the water surface could help alleviate contamination problems with the ostracod *Cypretta globulus* because the ostracod adheres to leaves that can be easily removed and replaced.[10]

A density-dependent relationship among juvenile insects is often directly associated with the availability of food resources and consequently with increased juvenile mortality, delayed maturity, and reduced adult size.[39] Addition of suitable supplementary food helped prolong the controlling efficacy of a combination of *M. aspericornis* and Bti in controlling *Ae. aegypti* mosquito larvae in peridomestic containers. Implementation of a combination of *M. aspericornis* and Bti with supplementary food provided by the local community to control *Ae. aegypti* mosquito larvae under field conditions should be investigated.

Received December 19, 2002. Accepted for publication May 29, 2003.

Acknowledgments: We thank Dr. Janet W. Reid for identification of copepod specimens, Dr. John R. Milne for reviewing the manuscript, Siriporn Vihokto for providing initial cultures of copepods, and Samnieng Thienthong and Kitti Thienthong for maintaining cultures of copepods and mosquitoes, respectively.

Financial support: This investigation was supported by the UNDP/World Bank/Worls Health Organization Special Program for Research and Training in Tropical Diseases (TDR/RCS/A00786) and the Thailand Research Fund (RTA/05/2540).

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World Journal of Microbiology & Biotechnology **19**: 903–906, 2003.

Isolation and characterization of pathogens attacking *Pomacea canaliculata*

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Received 6 January 2003; accepted 27 June 2003

Keywords: Biocontrol, isolation, LC_{50}, microbial control, *Pomacea canaliculata*

Summary

Seven microorganisms capable of killing *Pomacea canaliculata* were isolated from soil samples obtained from various agricultural areas of Thailand. The identification of these microorganisms was performed using microscopic examination and biochemical tests. Five strains were identified as *Pseudomonas aeruginosa* and were designated *P. aeruginosa* 19.1, 21.2.1, B1.1, P1 and P2. The other two strains were identified as *Pseudomonas fluorescens* and were designated *P. fluorescens* 13.1 and Ct1.

Pathogenicity studies of these microorganisms to *P. canaliculata* (Lamarck) were performed and characterized by LC_{50} levels. The LC_{50} levels of non-autoclave-treated and autoclave-treated cell suspensions to *P. canaliculata* were found to be 3.56×10^4–1.35×10^6 c.f.u./ml and 3.09×10^4 to 1.23×10^6 c.f.u./ml, respectively.

Introduction

Pomacea canaliculata is commonly known as the golden apple snail. It was introduced as a human food from Argentina to Taiwan in 1979. Unfortunately, the snails were not a commercial success and were found to transfer the *Angiostrongylus cantonensis* (rat lung worm) parasite to humans if not cooked enough before eaten (Fulton & Supanwong 1968; Keawjam 1986). Additionally, *P. canaliculata* is an intermediate host of the intestine fluke, *Echinostoma ilocanum*, which causes abdominal pain and diarrhoea in man. The snails are well adapted to tropical regions and they can survive in hostile environments. For example, they can hide in the mud during dry periods and in water with low oxygen content. They use a tubular siphon to breathe air while they are under water. The snails eat almost everything including all vegetables and dead animal life. Their fast growth and reproduction ensures that even a few snails quickly spread, especially in environments like rice fields. With only a few natural enemies to constrain them, the snails have rapidly developed into a serious pest in many areas with cultivated rice land such as Thailand, Cambodia, Indonesia, Hong Kong, Southern China, Japan, and the Philippines (Hirai 1988). To control this snail population, Asian rice farmers have tried a variety of responses to this infestation such as hand-picking snails, transplanting older rice seedlings and raising ducks in the paddy fields (Hirai 1988). Eventually, most rice farmers began using pesticides in an attempt to control the snails. In addition to being costly, most of the pesticides applied were not registered for use against mollusks in freshwater ecosystems, which resulted in serious repercussions for environmental and human health. Therefore, an appropriate snail control system should be identified. Recently, there have been reports that some strains of fluorescent pseudomonads colonize the roots of crop plants and protect them from diseases caused by soil-borne fungal pathogens when these bacteria are introduced into soil (Ursula *et al.* 2001). Therefore, we tried to isolate appropriate snail pathogens such as microorganisms which can be further used as biocontrol agents against *P. canaliculata* and which do not harm humans or fresh water ecosystems.

In this study, seven microorganisms capable of killing *P. canaliculata* were isolated and identified. The pathogenicity of these microorganisms to *P. canaliculata* was characterized by LC_{50} levels.

Materials and methods

Bacterial strains and media

The strains used in this study were *P. aeruginosa* 19.1, 21.2.1, B1.1, P1 and P2, and *P. fluorescens* 13.1 and Ct1 which are newly isolated *P. canaliculata* microbial pathogens. *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* were used as inactive controls because they are unable to kill *P. canaliculata*. For routine

cultivation of these microorganisms, the media used were nutrient agar and nutrient broth purchased from Oxoid (England). The strains were maintained as stab cultures in nutrient agar media at 4 °C or in glycerol at −20 °C, and then propagated twice at 30 °C before being used experimentally.

Snail and culture procedures

The snails used in all experiments were obtained as egg clutches from the pond at Kasetsart University, Thailand. The egg clutches were left above the waterline at room temperature until the young snails hatched and fell into the water. The water used for the snail cultivation was dechlorinated tap water left to stand for 18–24 h before use. The stock snails were fed the same food, papaya leaf. Snails were selected by size, not age. They were routinely cultured separately in 150 ml dechorinated water with a non-autoclave-treated cell suspension or autoclave-treated cell suspension inoculum at room temperature without food supplementation. The snail controls were run in parallel by culturing snails in dechlorinated water without any inoculum. The inactive controls were run by culturing snails in non-autoclave-treated and autoclave-treated cell suspensions of *E. coli*, *B. subtilis* and *S. aureus*.

Isolation of P. canaliculata *microbial pathogens*

Seven *P. canaliculata* microbial pathogens were isolated from a total of 200 soil samples obtained from various agricultural areas of Thailand. For the isolation procedure, samples were obtained by measuring 5 g soil and mixing it into 150 ml dechlorinated water in a plastic beaker. Ten *P. canaliculata* with 3–5 mm operculum diameter were put into the soil suspension. The death of snails was recorded daily for 30 days. Snail death was defined as no activity when pricking the snail's head or foot. All dead snails were removed from the soil suspension as soon as observed.

Identification of seven P. canaliculata microbial pathogens

To identify the seven newly isolated *P. canaliculata* microbial pathogens, the following properties were examined: cell morphology, Gram stain, spore formation, flagella staining, motility test, oxidase activity, pigment formation (pyocyanin, pyoverdin), oxidative-fermentative test of glucose, fructose, xylose, maltose, sucrose, lactose and manitol, ornithine decarboxylase, lysine decarboxylase, arginine decarboxylase, reduction of nitrate, citrate, hydrolysis of urea and production of indole. The inoculated media were incubated at 30 °C and read after 24 and 48 h.

Determination of lethal concentration killing 50% of the snails (LC_{50})

Pathogenicity to *P. canaliculata* of the seven strains prepared as untreated and autoclave-treated cell suspensions was studied. For preparation of the untreated cell suspension, the seven microorganisms were separately cultured on nutrient agar at 30 °C for 18–24 h. Then, the cells were harvested and inoculated into a 5000 ml sterilized beaker containing 4500 ml of sterilized dechlorinated tap water. Five millilitre aliquots of cell suspension were taken and used for the determination of viable cell counts using the standard spread plate technique. Then, the cell suspensions were prepared in five various cell concentrations from 10^7 to 10^3 c.f.u./ml by serial ten-fold dilutions. These cell concentrations covered the highest concentrations that killed most or all snails and the lowest concentrations that killed none or only a few snails, during the exposure period. Half the volumes of these cell suspensions were prepared as autoclave-treated cell suspension by autoclaving at 121 °C at 15 lb/in.^{2} pressure for 20 min. These various cell concentrations of untreated or autoclave-treated cell suspensions were further separately transferred into 15 plastic beakers for a final volume of 150 ml. One *P. canaliculata* snail per beaker was separately added to each of 15 beakers containing untreated cell suspension and to each of 15 beakers containing autoclave-treated cell suspension for each microorganism. Snail deaths were recorded at 24 h intervals over a 72 h period. The controls were run in parallel with active cell suspensions by adding snails to dechlorinated water without microbial inoculum. All LC_{50} tests included three replications at room temperature without food supplementation.

Statistical analysis

Probit's analysis was used to calculate the LC_{50} with 95% confidence. LC_{50} was defined as the concentration of colony-forming units per millilitre where 50% of laboratory animals died.

Results and discussion

Isolation and identification of seven P. canaliculata *microbial pathogens*

From 200 soil samples, dead snails were observed in 12 soil suspensions. These soil suspensions were separately streaked on nutrient agar to obtain pure cultures. Thirty pure cultures were obtained and further studied regarding their capabilities to kill *P. canaliculata*. Seven pseudomonads, namely, *Pseudomonas aeruginosa* 19.1, 21.2.1, B1.1, P1, P2, and *Pseudomonas fluorescens* 13.1 and Ct1 at 1.8×10^6 to 8.5×10^6 c.f.u./ml cell concentration were able to kill *P. canaliculata*. As illustrated in Table 1, no snail died after 24 h of incubation. Subse-

Table 1. The killing capabilities of non-treated and autoclave-treated cell suspensions of seven *Pomacea canaliculata* microbial pathogens.

Microorganism	Cell concentration (c.f.u./ml)	Preparation of cell suspension	Numbers of dead snails per total numbers of snails and percent of dead snails at various incubation times		
			24 h	48 h	72 h
P. aeruginosa 19.1	8.6×10^6	Non-treated	0/10 (=0%)	5/10 (=50%)	10/10 (=100%)
		Autoclave-treated	0/10 (=0%)	4/10 (=40%)	10/10 (=100%)
P. aeruginosa 21.2.1	3.7×10^6	Non-treated	0/10 (=0%)	2/10 (=20%)	9/10 (=90%)
		Autoclave-treated	0/10 (=0%)	2/10 (=20%)	9/10 (=90%)
P. aeruginosa B1.1	5.7×10^6	Non-treated	0/10 (=0%)	7/10 (=70%)	9/10 (=90%)
		Autoclave-treated	0/10 (=0%)	7/10 (=70%)	10/10 (=100%)
P. aeruginosa P1	1.8×10^6	Non-treated	0/10 (=0%)	6/10 (=60%)	10/10 (=100%)
		Autoclave-treated	0/10 (=0%)	5/10 (=50%)	10/10 (=100%)
P. aeruginosa P2	2.5×10^6	Non-treated	0/10 (=0%)	6/10 (=60%)	8/10 (=80%)
		Autoclave-treated	0/10 (=0%)	6/10 (=60%)	10/10 (=100%)
P. fluorescens 13.1	5.0×10^6	Non-treated	0/10 (=0%)	7/10 (=70%)	10/10 (=100%)
		Autoclave-treated	0/10 (=0%)	6/10 (=60%)	10/10 (=100%)
P. fluorescens Ct1	4.1×10^6	Non-treated	0/10 (=0%)	7/10 (=70%)	10/10 (=100%)
		Autoclave-treated	0/10 (=0%)	7/10 (=70%)	10/10 (=100%)
Bacillus subtilis (inactive)	6.0×10^6	Non-treated	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)
		Autoclave-treated	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)
E. coli (inactive)	5.8×10^6	Non-treated	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)
		Autoclave-treated	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)
S. aureus (inactive)	5.2×10^6	Non-treated	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)
		Autoclave-treated	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)
Control	0	No inoculation	0/10 (=0%)	0/10 (=0%)	0/10 (=0%)

quently however, 20–70% died after 48 h of incubation and 80–100% died after 72 h of incubation. In contrast, no snail controls died when simultaneously cultured in dechlorinated water without any microbial inoculum. Moreover, no dead snails resulted from inoculation with cell suspensions of inactive strains such as *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. In a previous report, the mortality of the adult tsetse fly, *Glossina morsitans morsitans*, caused by *P. aeruginosa* was studied and it was found that *P. aeruginosa* at 10^7 c.f.u./ml caused high mortality ranging from 70 to 98% at 8 days of post-ingestion (Kaaya & Darji 1989).

Identification of the seven *P. canaliculata* microbial pathogens was made by microscopic examination and biochemical tests. *P. aeruginosa* 19.1, 21.2.1, B1.1, P1 and P2 were Gram negative bacilli with one polar flagellum and no spore formation. For biochemical tests, positive results were found with the oxidase test, pyocyanin and pyoverdin formation, oxidative test of glucose, oxidative test of xylose, ornithine decarboxylase, lysine decarboxylase, arginine decarboxylase, nitrate reduction test, citrate production, urease test and motility test. On the other hand, negative results were found with tests of H_2S production, the indole test and oxidative tests of maltose, sucrose and lactose. Microscopic identification of *Pseudomonas fluorescens* 13.1 and Ct1 showed that these bacteria were Gram negative bacilli with more than one flagellum and no spore formation. Biochemical characteristics included positive results for the tests of oxidase, motility, pyoverdin, lysine decarboxylase, nitrate reduction and oxidative test of glucose, maltose and manitol and negative results for tests of H_2S production and the oxidative test of lactose. These bacteria showed K/N phenomenon on TSI media. Based on the identification scheme in *Bergey's Manual* (Holt *et al.* 1994), strains 19.1, 21.2.1, B1.1, P1 and P2 were identified as *Pseudomonas aeruginosa* and strains 13.1 and Ctl were identified as *Pseudomonas fluorescens*.

LC_{50} of seven P. canaliculata *microbial pathogens*

In order to obtain information concerning the pathogenicity of these seven microorganisms to *P. canaliculata*, LC_{50} levels of these seven microorganisms to *P. canaliculata* were studied as shown in Table 2. The LC_{50} levels after 72 h of non-autoclave-treated and autoclave-treated cell suspension snail exposure ranged from 3.56×10^4 to 1.35×10^6 and 3.09×10^4 to 1.23×10^6 c.f.u./ml, respectively. It is worth noting that *P. aeruginosa* 21.2.1 was found to be the most pathogenic strain for *P. canaliculata*. This bacterium may be the most potent strain for practical biocontrol of the snail pest. It is unclear why autoclave-treated cell suspensions of these seven isolates were also toxic to laboratory animals. However, this phenomenon suggests that reactive components which were capable of killing *P. canaliculata* were heat stable toxins or were not proteins. As shown in previous studies, *P. fluorescens* can produce a variety of secondary metabolites, in particular the antibiotics pyoluterorin, 2–4-diacylphloglucinol and hydrogen cyanide, which can protect various plants from diseases caused by soil-borne pathogenic fungi when introduced into soil (Schnider 1995; Laville *et al.* 1998). As well as the pathogenicity of *P. aeruginosa*, an

Table 2. LC_{50} levels after 72 h for seven *Pomacea canaliculata* microbial pathogens prepared as non-treated and autoclave-treated cell suspensions for *Pomacea canaliculata*.

Microorganism	Cell suspension preparation	LC_{50} levels for *Pomacea canaliculata* (c.f.u./ml)
P. aeruginosa 19.1	Non-treated	1.35×10^6
	Autoclave-treated	1.23×10^6
P. aeruginosa 21.2.1	Non-treated	3.56×10^4
	Autoclave-treated	3.09×10^4
P. aeruginosa B1.1	Non-treated	9.37×10^5
	Autoclave-treated	1.04×10^6
P. aeruginosa P1	Non-treated	9.34×10^4
	Autoclave-treated	8.42×10^4
P. aeruginosa P2	Non-treated	1.71×10^5
	Autoclave-treated	1.48×10^5
P. fluorescens 13.1	Non-treated	1.74×10^5
	Autoclave-treated	1.87×10^5
P. fluorescens Ct1	Non-treated	1.45×10^5
	Autoclave-treated	1.23×10^5

opportunistic pathogen may be a consequence of its ability to produce a wide variety of both cell-associated and extracellular virulence factors. Cell-associated virulence factors include pili, flagella, lipopolysaccharide, a type III secretion system, and alginate. Secreted products include low-molecular-weight toxins, such as phenazines, rhamnolipid, and cyanide, and numerous protein virulence factors, including ADP-ribosylating enzymes, proteases, and phospholipases (Choi *et al.* 2002). *P. aeruginosa* is widely distributed in the natural environment and can also act as a pathogen for plants and a variety of additional non-vertebrate hosts including *Czaenorhabditis elegans*, *Drosophila melanogaster*, and the greater wax moth, *Galleria mellonella* (Miyata *et al.* 2003). However, from our review, there is no information concerning *P. canaliculata* microbial pathogens. Therefore, we suggest that the purification and identification of reactive components of these *P. canaliculata* pathogens should be further studied. Further, the impact of intact cells of these bacteria on freshwater ecosystems should be investigated in field studies. Purified reactive components may be of use in microbial control of *P. canaliculata* in the future.

Acknowledgement

We are grateful to Dr Stephen Hamann, Faculty of Medicine, Rangsit University for editing this manuscript.

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CURRENT MICROBIOLOGY Vol. 48 (2004), pp. 175–181
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Current Microbiology
An International Journal

Expression of Chitinase-Encoding Genes in *Bacillus thuringiensis* and Toxicity of Engineered *B. thuringiensis* subsp. *aizawai* Toward *Lymantria dispar* Larvae

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Received: 29 March 2003 / Accepted: 16 June 2003

Abstract. Chitinase genes from *Aeromonas hydrophila* and *Bacillus circulans* No.4.1 were cloned into the plasmid pHY300PLK and designated as pHYA2 and pHYB43, respectively. Both plasmids were introduced into various strains of *B. thuringiensis* by electroporation. Plasmid pHYB43 was generally structurally stable, but showed lower segregrational stability than pHYA2 in *B. thuringiensis* subsp. *aizawai* when grown under nonselective conditions. The production of chitinase from *B. thuringiensis* subsp. *aizawai* harboring pHYB43 or pHYA2 could be detected after native polyacrylamide gel electrophoresis by using 4-methylumbelliferyl β-D-*N,N′*- diacetylchitobioside as the substrate. Moreover, *B. thuringiensis* subsp. *aizawai* harboring pHYB43 gave 15 times higher chitinase activity than when harboring pHYA2, as determined by means of a colorimetric method using glycol chitin as the substrate. In addition, *B. thuringiensis* subsp. *aizawai* harboring pHYB43 was more toxic to gypsy moth larvae (*Lymantria dispar*) than parental *B. thuringiensis* subsp. *aizawai* or its clone harboring pHYA2.

The strains of *Bacillus thuringiensis* (*B.t.*) produce toxins with highly selective modes of action [1, 5], such that the toxicity of a particular isolate may be restricted to very specific target insects. Attempts have been made by various groups to increase *B.t.* effectiveness for controlling insect pests. For example, we have attempted to improve the efficacy of insect killing by *B.t.* subsp. *israelensis* strain c4Q272 by introducing recombinant plasmids containing chitinase genes from *Aeromonas hydrophila* or *Pseudomonas maltophilia.* However, the introduced chitinase genes were unstable because of resulting deletions [21].

The addition of chitinase to *B.t.* preparations to enhance their effectiveness against spruce budworm and diamondback moths has been reported [9, 11, 14, 15, 21]. Also found was that addition of chitinolytic bacteria isolated from the midgut of *Spodoptera littoralis* to *B.t.* subsp. *entomocidus* increased the mortality of second instar larvae from 12% to 100% [17]. Recently, Sampson and Gooday reported that the chitinase of *B.t.* was involved in its pathogenicity to insects [13]. It has been proposed that chitinase destroys chitinous elements in the insect gut and thus allows for an increased bacterial content in the gut epithelium [2].

The purpose of this study was to construct recombinant plasmids containing chitinase-encoding genes from *A. hydrophila* and *B. circulans* No.4.1, and to introduce these recombinant plasmids into strains of *B.t.* The stability and toxicity of bacterial clones carrying these recombinant plasmids was studied with gypsy moth (*Lymantria dispar*) larvae.

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Materials and Methods

Plasmids, bacterial strains, and media. *Escherichia coli* DH5α was purchased from Gibco BRL, USA. *Bacillus subtilis* ISW1214 was obtained from Takara Shuzu, Japan. DHD provided *B. thuringiensis* subspecies. All of them were used as the cloning hosts for recombinant plasmids. They were grown at 37°C in Luria-Bertani (LB) medium with the appropriate antibiotic required for plasmid or strain selection

Table 1. Microorganisms and plasmids used in this study

Bacteria	Description	Source
Escherichia coli DH5α	*endA1 hsdR17 supE44 thi-1 gyrA relA1*$^{\Delta}$*(lacZYA-argF) U169 deoR* [$^{\phi}$*80 dlac*$^{\Delta}$*(lacZ) M15]*	Gibco BRL
Bacillus thuringiensis		
subsp. *israelensis* strain 4Q2	Serot-14	
strain 4Q272	bears only 72 mDal plasmid, Serot-14	
strain c4Q272	plasmidless	DHD
strain A084	streptomycin resistance, Serot-14	
subsp. *kurstaki*	Serot-3a3b	
subsp. *aizawai*	Serot-7	
Plasmids	Description	Sources
pHY300PLK	shuttle vector, *E. coli-B. subtilis*, ampicillin- and tetracycline resistance	Takara, Japan
pJP2514	pUC12 containing 3.9-kb chitinase gene of *Aeromonas hydrophila*	Prof. J.M. Pemberton
pCHIB1	pBluescript KS containing 2.6-kb chitinase gene of *B. circulans* No.4.1	Our laboratory
pHYA2	pHY300PLK containing chitinase gene from pJP2514	This study
pHYB43	pHY300PLK containing chitinase gene from pCHIB1	This study

[e.g., ampicillin (Ap) at 100 μg/mL and tetracycline (Tc) at 15 μg/mL]. For the solid media, 1.5% agar (Difco) was added to the liquid medium.

All pure cultures of bacteria were kept as stock cultures in 15% glycerol at −70°C. Bacterial strains and plasmids used in this study are listed in Table 1.

Construction of recombinant chitinase gene. The chitinase genes from *A. hydrophila* (pJP2514) (a gift from Prof. J.M. Pemberton, Queensland University, Australia) and *B. circulans* No. 4.1 (pCHIB1) from our laboratory were used in this study. The recombinant plasmid pJP2514 contained a 3.9-kb insert of the chitinase gene from *A. hydrophila*, which had been cloned into *E. coli* with pUC12 as the cloning vector [12]. The chitinase gene from *B. circulans* No.4.1 was subcloned into pBluescript KS as a 2.6-kb chitinase fragment and designated as pCHIB1 (our laboratory). It synthesized a chitinase of molecular mass 66 kDa. Both of chitinase genes expressed genes from theirs own promoter. The presence of promoter was examined by reversing the orientation of the chitinase gene in both pBluescript KS and SK. All of the plasmids could express chitinolytic activity.

Construction of pHYA2 (8.8 kb). pJP2514 and shuttle vector pHY300PLK were doubly digested with *Eco*RI-*Sal*I (ES). The chitinase gene (3.9 kb) from the pJP2514 digest (Fig. 1A) was separated by 0.7% agarose gel electrophoresis (Seakem GTG, FMC), and the ES fragment was recovered from the gel slice by using an agarose gel extraction kit (QiaexII, Qiagen Inc.). It was then ligated with linearized pHY300PLK (Fig. 1B) at an ES site.

Construction of pHYB43 (7.5 kb). pCHIB1 was digested with *Hin*dIII and the digest separated by 0.7% agarose gel electrophoresis. A 2.6-kb *Hin*dIII fragment containing the chitinase gene from pCHIB1 (Fig. 1 C) was recovered from the gel with an agarose gel extraction kit (QiaexII). It was then ligated with *Hin*dIII-digested pHY300PLK (Fig. 1B). The ligated products were subsequently transformed into *E. coli* DH5α. The presence and orientation of chitinase genes in transformants were confirmed by restriction endonuclease digestion and Southern hybridization. The intact plasmids, pHYA2 and pHYB43 (Fig. 1D), were then extracted from an *E. coli* DH5α clone and transformed into *Bacillus* spp., approximately 10^7 cfu (OD600~0.15), by electroporation (Gene Pulser II, BioRad), by using a capacitance of 25 μF, 400 Ω and 2.3 kV, single pulse.

Southern blot hybridization. Plasmids were separated by agarose gel electrophoresis. Gels were then soaked in 0.25 M HCl for 15 min, denatured in 0.5 M NaOH for 30 min, and rinsed with deionized distilled water. DNA fragments were transferred to a nylon membrane by using a Vacuum Blotter (Model 785, BioRad, USA) for 90 min. The membrane was then washed with 2 × SSC, dried, crosslinked with UV crosslinker (GS Gene Linker UV Chamber, BioRad), and probed with digoxigenin-11-dUTP-labeled chitinase gene(s). For DNA probes, the ES fragment from recombinant pJP2514 and the *Hin*dIII fragment from recombinant pCHIB1 were labeled with digoxigenin-11-dUTP-labeled DNA by the random prime method (labeling and detection of hybrid DNA by enzyme immunoassay as described by the manufacturer, Boehringer Mannheim). After hybridization, the membrane was washed 2× for 5 min in SSC, 0.1% SDS at room temperature, and then 2× for 15 min in 0.1 SSC, 0.1% SDS at 68°C under constant agitation. Immunological detection was done as described by the manufacturer (Boehringer Mannheim).

Determination of chitinase activity. Expression of chitinase genes in various hosts was determined by observation of the hydrolytic zone on colloidal chitin agar, by using a colorimetric method, with detection of a fluorescent substrate, 4-methylumbelliferyl-β-D-N,N′-diacetylchitobioside [4-MUF$(GlcNAc)_2$] (Sigma-Aldrich Inc., USA).

Colloidal chitin agar. Colonies of transformants were spotted on colloidal chitin agar (1.5% nutrient agar containing 0.3% colloidal chitin) and incubated at 37°C for 10 days. Hydrolytic zones around the colonies were measured.

Colorimetric method. Chitinase activity was determined by measuring the reducing end group, N-acetylglucosamine (NAG), degraded from colloidal chitin as described by Imoto and Yagishita [8]. The assay mixture consisted of 0.1 mL of enzyme solution, 0.1 mL of 0.01% glycol chitin, and 0.2 mL of 0.1 M McIlvaine buffer, pH 6.0. After incubation at 37°C for 15 min, 2.0 mL of 1.5 mM potassium ferricyanide reagent was added. The reaction was terminated by heating in boiling water for 15 min. The clear solution was measured immediately in a spectrophotometer at 420 nm. Activity was calculated by using a standard curve obtained by using known concentrations of NAG (0–0.15 mg). Chitinase activity was calculated and expressed as mU/mL of original volume of cell cultures. One unit of activity was defined as the

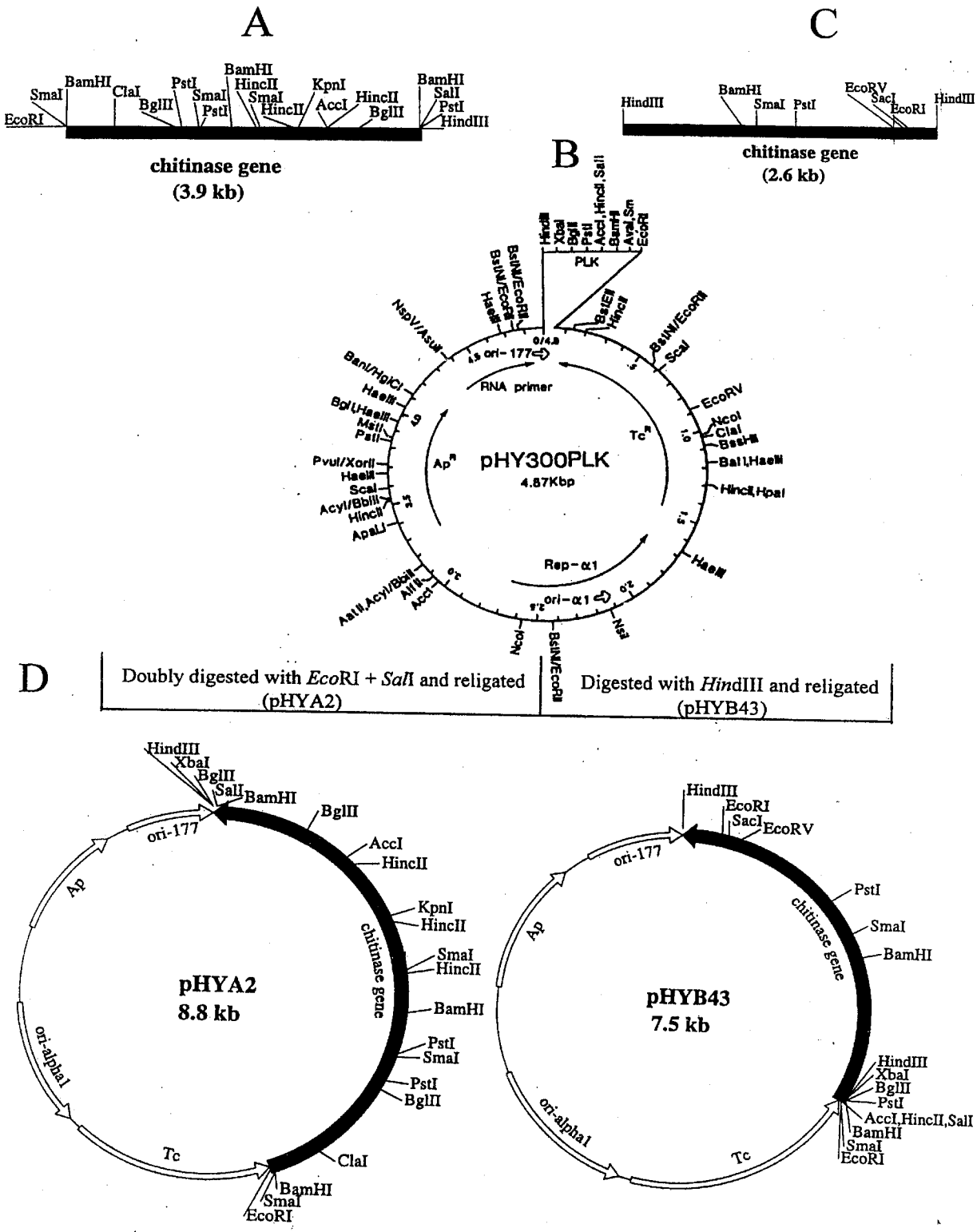

Fig. 1. The construction of pHYA2 and pHYB43. Restriction maps of the chitinase gene from *A. hydrophila*, the shuttle vector pHY300PLK, and the chitinase gene from *B. circulans* No. 4.1 are shown in **A, B,** and **C,** respectively. The maps of pHYA2 and pHYB43 carrying 3.9-kb and 2.6-kb chitinase gene fragments are illustrated in **D.** All DNA fragments above were isolated from agarose gel. The chitinase genes were inserted into shuttle vector pHY300PLK at an ES site for pHYA2 and at a *Hin*dIII site for pHYB43. The resulting vectors were used to transform *E. coli* DH5α. Suspected transformants carrying plasmid pHYA2 and pHYB43 were confirmed by restriction mapping.

amount of enzyme that liberated 1 μmole of NAG per min under standard assay conditions. Negative control tubes contained all components except substrate, and blanks contained all components except the enzyme.

Using the fluorescent substrate. Colonies of transformants were exposed to chloroform vapor for 20 min and overlaid with soft agar (0.7% agar) containing 0.05 μg/mL of 4-MUF$(GlcNAc)_2$. Light-blue fluorescence of methylumbelliferone released was observed by UV light at 365 nm.

Chitinase activity was also detected in acrylamide gels by using a fluorescent substrate. *B.t.* subsp. *aizawai* host and its clone harboring plasmids were separately cultured in 40 mL of LB broth and LB broth containing Tc to delay the sporulation phase of growth produced large amounts of chitinase. All cultures were incubated at 37°C on a rotary shaker at 200 rpm, overnight. The supernatant and pellets were separated by centrifugation (*Hettich* Zentrifugens) at 5000 rpm and 4°C for 30 min. The supernatant was concentrated with a concentrator tube (Ultrafree®, Millipore), and pellets were resuspended in 1 mL of sterile distilled water for sonication (Soniprep 150). Both the supernatant and sonicated cells were analyzed by native polyacrylamide gel electrophoresis (PAGE) at 100 V for approximately 90 min. The gel was overlaid with soft agar (0.7% agar) containing 0.05 μg/mL of 4-MUF-$(GlcNAc)_2$ and was incubated at 37°C for 15 min. The light-blue fluorescence of methylumbelliferone release could be observed under UV light at 365 nm to localize chitinase activity bands. Crude chitinase from *E. coli* carrying pJP2514 was used as a positive control.

Stability test. The stability of plasmids was examined by growing *B.t.* subsp. *aizawai* harboring pHYA2 or pHYB43 in LB broth with daily transfer to fresh LB broth without antibiotic for 30 days. On certain days, 0.1-mL samples from a 10-fold serial dilution were then plated on LB agar with and without Tc, and the number of colonies was counted. Tetracycline-resistant colonies were recorded as a percentage of the total colony forming units (cfu) on LB without Tc [i.e., % tetracycline-resistant colonies = (tetracycline-resistant cfu/total cfu) × 100].

Bioassays of insecticidal activity.

Preparation of gypsy moth larvae. DHD as providing the gypsy moth's cocoons. They were hatched by being incubated at 30°C with 100% moisture for 1 day. The newly hatched gypsy moth (*L. dispar*) larvae were used for bioassays of insecticidal activity.

Preparation of B.t. spores and chitinase. Parental *B.t.* subsp. *aizawai* was grown in LB broth, and transformants of *B.t.* subsp. *aizawai* harboring pHYB43 or pHYA2 were grown separately in LB broth with Tc at 30°C with vigorous shaking overnight to delay the sporulation phase of growth for increasing the chitinase production. Cultures were then collected separately, and supernatants and cells were separated by centrifugation at 12000 rpm for 30 min at 4°C. The culture supernatants were kept at −80°C, whereas the cells were spread separately on Schaeffer sporulation medium (SSM) plates, incubated at 30°C for 4 days, and then scraped off to collect the spores for resuspension in 1 mL sterile deionized water at a concentration of 30 mg/mL. The suspensions were boiled at 80°C for 15 min to kill vegetative cells, then diluted to 10^6 cfu by culture supernatants (chitinase) used as stock mixture.

Preparation of the mixtures on diet cube. A 100-μl aliquot from the stock mixture, containing 10^5 cfu of bacterial stock plus 1.5 mU chitinase, was soaked into each cube of artificial diet [2% Agar N.F. 9009-18-0 (ICN, USA) containing 160 g/L of gypsy moth diet (Bio-serv. Inc.)], 2 cm in diameter and 1 cm in depth. After air drying for 2 h, ten newly hatched gypsy moth larvae were put onto each cube and incubated at 25°C. Experiments were done in triplicate, and mortalities were recorded every 12 h for 5 days as a percentage of mortality of insect larvae: % mortality = (number of dead larvae/total number of larvae) × 100. The control group was fed the same diet soaked with LB culture broth.

Results

Construction of recombinant plasmids carrying cloned chitinase genes in the shuttle vector pHY300PLK. The presence of plasmids pHYA2 and pHYB43 in *E. coli* DH5α clones was confirmed by restriction endonuclease digestion and Southern hybridization. This demonstrated that the chitinase genes from *A. hydrophila* and *B. circulans* No.4.1 were present in the new constructs. These transformants could hydrolyze colloidal chitin and 4-MUF$(GlcNAc)_2$ under the same conditions as the original source clones (data not shown).

Expression of chitinase gene in various hosts. The plasmids pHYA2 and pHYB43 obtained from *E. coli* DH5α transformants were transformed into *B.t.* subsp. *aizawai.* The six colonies of each transformants were randomly selected. It was shown that recombinant plasmid pHYA2 was not structurally stable. It appeared that a part of the DNA was deleted. Therefore, attempts were made to study the fate of pHYA2 in various *B.t.* strains, i.e., *B.t.* subsp. *israelensis* strain 4Q2, 4Q272, c4Q272, A084 and subsp. *kurstaki.* The results indicated that a part of pHYA2 DNA was deleted in all these hosts. By contrast, pHYB43 was structurally stable in *B.t.* subspecies. By means of native-PAGE using 4-MUF$(GlcNAc)_2$ as the detection substrate, chitinase activity could be detected in the culture supernatant from all samples of the *B.t.* subsp. *aizawai* host (Fig. 2B, lane 2) harboing pHYB43 (Fig. 2B, lane 3) or harboring pHYA2 (Fig. 2B, lane 4), but in the sonicated cells of only *B.t.* subsp. *aizawai* harboring pHYB43 (Fig. 2D, lane 3). Interestingly, the transformants of *B.t.* subsp. *aizawai* produced only heterologous chitinase, not endogenous chitinase. The molecular masses of the chitinases from the transformants were smaller (pHYB43) and larger (pHYA2) than those of the untransformed hosts (Fig. 2B, lane 2–4). *B.t.* subsp. *aizawai* carrying pHYB43 gave the highest chitinase activity in the culture medium. This was approximately 15 mU/mL, while that of *B.t.* subsp. *aizawai* carrying pHYA2 was 1 mU/mL when detected by the colorimetric method with glycol chitin as the substrate.

Segregrational stability of recombinant plasmids in *B.t.* subsp. *aizawai.* Serial analysis of plasmids pHYB43 and pHYA2 in *B.t.* subsp. *aizawai* for 30 passages showed that pHYB43 was retained in *B.t.* subsp. *aizawai* in medium without antibiotic for approximately

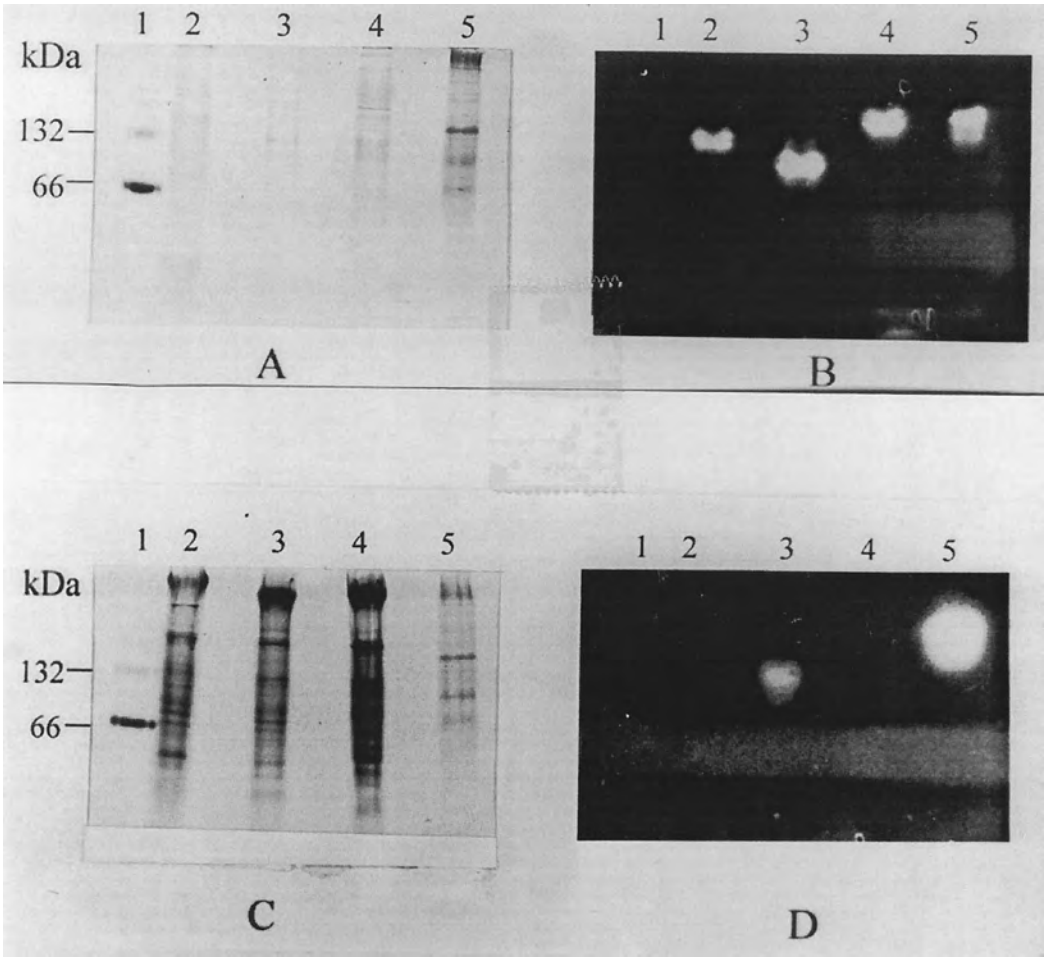

Fig. 2. Native-polyacrylamide gel electrophoresis for detection of chitinase activity from *B.t.* subsp. *aizawai* transformants harboring plasmid pHYA2 or pHYB43. **A.** Gel patterns for proteins from supernatants. Lane 1, bovine serum albumin (BSA: monomer, 66 kDa; dimer, 132 kDa); lane 2, *B.t.* subsp. *aizawai* host; lane 3, *B.t.* subsp. *aizawai* harboring pHYB43; lane 4, *B.t.* subsp. *aizawai* harboring pHYA2 and lane 5, *E. coli* harboring pJP2514. **B** shows the corresponding chitinase activity. **C.** Gel patterns for protein from sonicated cells. Lane 1, BSA; lane 2, *B.t.* subsp. *aizawai* host; lane 3, *B.t.* subsp. *aizawai* harboring pHYB43; lane 4, *B.t.* subsp. *aizawai* harboring pHYA2; and lane 5, *E. coli* harboring pJP2514. **D** shows the corresponding chitinase activity.

three passages. It could be retained for more passages in medium supplemented with Tc. Plasmid pHYA2 was maintained in *B.t.* subsp. *aizawai* host for at least 30 passages.

Toxicity against *L. dispar*. Gypsy moth larvae (*L. dispar*) bioassays of parental and transformed *B.t.* subsp. *aizawai* harboring chitinase genes (pHYA2 or pHYB43) are shown graphically in Fig. 3 as the percentage of dead larvae in 5 days. Transformed *B.t.* subsp. *aizawai* harboring pHYB43 gave the highest toxicity (27 $\pm$ 3%), and this was significantly higher ($P < 0.05$) than the mortality from the parental strain by *t*-test statistical value. By contrast, clones harboring pHYA2 were not significantly different from parental *B.t.* subsp. *aizawai* at 17 $\pm$ 2% ($P > 0.05$). The larvae could survive on a diet containing culture broth (control group).

Discussion

The expression of chitinase-encoding genes from *A. hydrophila* and *Pseudomonas maltophilia* in transformed *B.t.* subsp. *israelensis* has been reported, but only low levels of expression were described, and the genes were structurally unstable in the hosts [21]. Here, expression of the chitinase gene from *A. hydrophila* in pHYA2 was low, and the plasmid was structurally unstable in *Bacillus* spp. By contrast, the chitinase gene from *B. circulans* No.4.1, pHYB43, was structurally stable in the hosts. It might be assumed that the deletion of the gene in *B.t.* strains might also be due to the modification and restriction system or site-specific endonucleases in *Bacillus* spp. The position 45 of *B. subtilis* chromosome possesses this property [7], and it may occur in *B.t.* strains. The

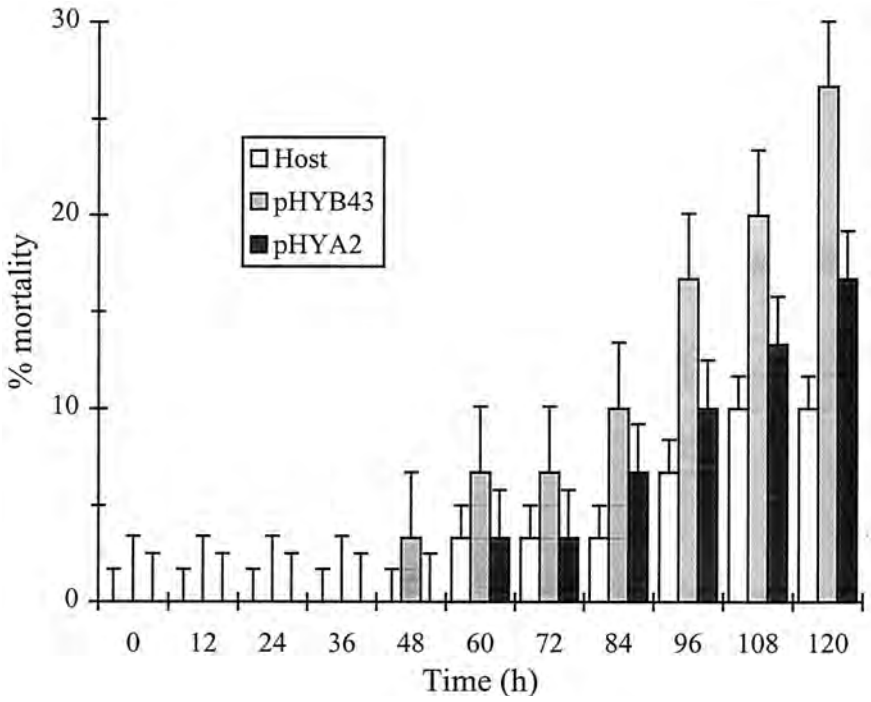

Fig. 3. Effect of parental and transformed *B.t.* subsp. *aizawai* harboring chitinase genes at the concentration of 10^5 cfu/diet on newly hatched gypsy moths (*L. dispar*). The results are presented graphically as the percentage mortality every 12 h for 5 days. Error bars show standard error (SE).

chitinase production from both pHYA2 and pHYB43 was too low to detect by using colloidal chitin. With these low activity clones, 4-MUF$(GlcNAc)_2$ was the most sensitive detection reagent and more useful than colloidal chitin. It required a smaller amount of enzyme, and a shorter time to complete hydrolysis [6, 20]. Plasmid pHYA2 contained a chitinase gene isolated from *A. hydrophila*, a Gram-negative bacterium. Therefore, the expression and/or the stability of this gene in a Gram-positive *Bacillus* may be compromised [18]. For example, a chitinase gene from *B. licheniformis* was retained in *B.t.* [19], whereas, chitinase genes from *P. maltophilia* or *A. hydrophila* were not [21]. However, in either case, only small amounts of chitinase were produced [19, 21].

The reduction in plasmid segregrational stability of pHYB43 expressed a higher amount of chitinase production than pHYA2 by a factor of 15 and under nonselective conditions may be a result of selective pressure against cells expressing the chitinase protein. The accumulation of heterologous protein in bacteria is often toxic for the host cell and can result in the loss of plasmid from a culture, a reduction in the amount of recombinant protein being expressed, or a decrease in culture viability [10]. In addition, the instability of the recombinant constructs can be affected by temperature, pH of the medium, aeration, composition of the growth medium, and the dilution rate [3].

The larvae control group were fed the diet soaked with LB culture broth. There was not an adverse effect on the larvae. This might be due to dose dependency or be affected by the temperature [11, 15, 19]. However, the ability of chitinase to increase the killing rate by *B.t.* has been reported by many authors using a variety of chitinase preparations such as crude chitinase, commercial chitinase, and even of chitinolytic bacteria extracted from the insect gut [9, 14, 15, 16, 17, 21]. These preparations of chitinase will have contained contaminating enzymes, such as proteases, which may also enhance insecticidal activity [13]. Interestingly, either clone harboring pHYB43 or pHYA2 produced the chitinase and secreted it into the culture supernatant (Fig. 2). They exhibited decreased and/or inhibited expression of the endogenous chitinase gene of the host (Fig. 2B). This may explain that both endogenous and heterologous chitinase-encoding genes use the same machinery to express chitinase genes, which may be titrated when the heterologous chitinase genes were cloned into the host. Moreover, it may result from the vector copy number, which is similar to Gamel and Piot [4]. They reported that the higher copy number of shuttle vector would increase the expression of heterologous genes but decrease the expression of endogenous genes. Therefore, the exogenous bacterial chitinase used in this study was a single enzyme, chitinase from *B. circulans* No.4.1 (pHYB43) or *A. hydrophila* (pHYA2), expressed in *B.t.* subsp. *aizawai.* Interestingly, *B.t.* subsp. *aizawai* harboring pHYB43 had higher toxicity than parental *B.t.* subsp. *aizawai* or its clone harboring pHYA2. It might be syn-

ergistically affected by various types of Cry1 proteins, found in *B.t.* subsp. *aizawai*, and chitinase [19]. Although the slightly higher toxicity was improved, the chitinase appeared to be responsible for the increased toxicity of the transformed *B.t.* subsp. *aizawai*. It is possible that future work on over-expression of a chitinase gene in a heterologous host, by using a plasmid with a replication origin coming from a natural plasmid from the host or by using a promoter working in a heterologous host instead of the promoter of the gene, may improve *B.t.* toxicity against insect larvae.

ACKNOWLEDGMENTS

This research work was supported by the Thailand Research Fund for the Senior Scholar Grant No. RTA4080005. We thank Prof. Timothy Flegel for critical reading of this manuscript.

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