DIVERSITY OF POLYHYDROXYALKANOATES-PRODUCING BACTERIA ISOLATED FROM CASSAVA PULP

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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ความหลากหลายของแบคทีเรียที่สร้างพอลิไฮดรอกซิแอลคาโนเอตซึ่งแยกได้จากกากมันสำปะหลัง

นางอรอุมา จันทร์เสถียร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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อรอุมา จันทร์เสถียร : ความหลากหลายของแบคทีเรียที่สร้างพอลิไฮดรอกซิแอลคาโนเอต ซึ่งแยกได้จากกากมันสำปะหลัง (DIVERSITY OF POLYHYDROXYALKANOATES-PRODUCING BACTERIA ISOLATED FROM CASSAVA PULP) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุรีลักษณ์ รอดทอง, 200 หน้า.

พอลิไฮดรอกซิแอลคาโนเอต (พีเอชเอ) เป็นพอลิเมอร์ชีวภาพกลุ่มหนึ่งที่เซลล์จุลินทรีย์ใช้เป็นแหล่งคาร์บอนและแหล่งพลังงานสำรอง พีเอชเอบางอนุพันธ์สามารถใช้เป็นพลาสติกย่อยสลายได้ทางชีวภาพ เนื่องจากมีสมบัติคล้ายคลึงกับปิโตรเคมีพอลิเมอร์ในอุตสาหกรรมพลาสติกที่ไม่สามารถย่อยสลายได้ และคาดว่าจะสามารถใช้ทดแทนปิโตรเคมีพอลิเมอร์เหล่านั้นได้ แบคทีเรียหลายชนิดสามารถสังเคราะห์พีเอชเอในสภาวะที่มีแหล่งคาร์บอนในปริมาณมากเกินพอในอาหารเลี้ยงเชื้อและมีสารอาหารที่จำเป็นในปริมาณจำกัด การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อหาความหลากหลายของแบคทีเรียที่สร้างพีเอชเอซึ่งแยกได้จากกากมันสำปะหลัง โดยตรวจหาสารพีเอชเอที่สะสมภายในเซลล์ และเปรียบเทียบเพื่อจัดกลุ่มของแบคทีเรียตามระดับการสะสมพีเอชเอ พร้อมทั้งศึกษาสัณฐานวิทยาของเซลล์ จากการศึกษาพีเอชเอที่สะสมภายในเซลล์แบคทีเรียด้วยเทคนิค Nile blue A และติดตามการสะสมสารด้วยวิธี Nile red ของแบคทีเรียจำนวน 772 ไอโซเลต พบแบคทีเรีย 82 ไอโซเลต ที่สามารถสะสมสารพีเอชเอในระดับโดยเฉลี่ยร้อยละ 3.3 ถึง 85.77 ของพื้นที่ทั้งหมดภายในเซลล์เมื่อตรวจสอบด้วยกล้องจุลทรรศน์ฟลูออเรสเซนต์ภายหลังจากการเลี้ยงเชื้อบนอาหารสมบูรณ์ที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 48 ชั่วโมง และย้ายลงเลี้ยงบนอาหารที่มีสารอาหารขั้นต่ำสูตรดัดแปลงที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลาอีก 48 ชั่วโมง จากนั้นระบุชนิดของแบคทีเรียที่สะสมพีเอชเอเหล่านี้ด้วยลักษณะทางสัณฐานและสรีรวิทยา และลำดับนิวคลีโอไทด์ของ 16S ribosomal RNA gene จากสัณฐานวิทยาของเซลล์สามารถแบ่งแบคทีเรียได้เป็น 4 กลุ่ม คือ กลุ่มแบคทีเรียแกรมบวกรูปร่างเซลล์เป็นท่อนไม่สร้างเอนโดสปอร์ กลุ่มแบคทีเรียแกรมลบรูปร่างเซลล์เป็นท่อน กลุ่มแบคทีเรียแกรมบวกรูปร่างเซลล์เป็นท่อนสร้างเอนโดสปอร์ และกลุ่มแบคทีเรียแกรมบวกรูปร่างเซลล์กลม ซึ่งมีจำนวน 15 3 59 และ 5 ไอโซเลต ที่สามารถสะสมสารพีเอชเอคิดเป็นร้อยละ 3.3-70.72 5.1-21.01 8.3-85.77 และ 51.9-74.99 ของพื้นที่ทั้งหมดภายในเซลล์ตามลำดับ เมื่อศึกษาสมบัติทางสรีรวิทยาของแบคทีเรียทั้ง 82 ไอโซเลต พบความหลากหลายของสกุลของแบคทีเรีย จำนวน 8 สกุล คือ *Bacillus* *Chryseobacterium* *Enterobacter* *Klebsiella* *Listeria* (ชนิดที่ไม่เป็นเชื้อก่อโรค) *Micrococcus* *Pseudomonas* และ *Staphylococcus* จากนั้นได้เลือกไอโซเลตตัวแทนของแบคทีเรียมาวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S ribosomal RNA gene ซึ่งได้ผลลำดับนิวคลีโอไทด์ในช่วง 1,381-1,439 คู่เบส ที่เมื่อเปรียบเทียบความเหมือนกับข้อมูลจาก

ฐานข้อมูล GenBank แล้วพบว่าแบคทีเรียแกรมลบรูปร่างเซลล์เป็นท่อนจำนวน 3 ไอโซเลต มีความเหมือนกับ *Pseudomonas aeruginosa* PAON 2 *Klebsiella oxytoca* SB 9 *Enterobacter hormaechei* CIP 103441 ^{T} ร้อยละ 96.7 99.1 และ 99.5 ตามลำดับ แบคทีเรียแกรมบวกรูปร่างเซลล์เป็นท่อนสร้างเอนโดสปอร์จำนวน 8 ไอโซเลต มีความเหมือนกับ *Bacillus megaterium* ATCC 14581^{T} *Bacillus cereus* ATCC 21281^{T} *Bacillus* sp. NCCP-158^{T} *B. cereus* ATCC 43881^{T} *B. subtilis* KQC 85 *Bacillus* sp. IMT 21^{T} *B. cereus* JS-33 และ *Bacillus* sp. PBCC 10^{T} ร้อยละ 99.9 99.2 99.7 98.0 99.6 99.2 89.8 และ 99.9 ตามลำดับ และตัวแทนของแบคทีเรียกลุ่มแกรมบวกรูปร่างเซลล์กลม 1 ไอโซเลต มีความเหมือนกับ *Staphylococcus cohnii* ATCC 29974^{T} ร้อยละ 99.3 แบคทีเรียที่แยกได้จากกากมันสำปะหลังที่ย่อยแป้งได้ดีและสะสมสารพีเอชเอได้มากกว่าร้อยละ 80 ของพื้นที่ทั้งหมดภายในเซลล์ 2 ไอโซเลต ที่ระบุชนิดได้ว่าคือ *Bacillus megaterium* มีศักยภาพในการใช้ประโยชน์เพื่อผลิตพอลิเมอร์ชีวภาพ

สาขาวิชาชีววิทยา
ปีการศึกษา 2553

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

ONUMA CHANSATEIN : DIVERSITY OF POLYHYDROXYALKANOATES-PRODUCING BACTERIA ISOLATED FROM CASSAVA PULP. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 200 PP.

DIVERSITY/POLYHYDROXYALKANOATES/BACTERIAL IDENTIFICATION/ POLYHYDROXYALKANOATES-PRODUCING BACTERIA

Polyhydroxyalkanoates (PHAs) is a group of biopolymers functioning as carbon and energy reserves in microbial cells. Some PHA derivatives can be served as biodegradable plastics that have properties similar to petrochemical polymers, non-degradable plastics, and are expected as the replacement for these petrochemical polymers. A wide range of bacterial species synthesize the polymers when a carbon source is provided in excess and essential growth nutrients are limited. The aim of this study was to determine the diversity of PHAs-producing bacteria isolated from cassava pulp samples. Seven hundred and seventy two bacterial isolates were investigated for PHA accumulation in their cells using Nile blue A technique as well as monitoring by Nile red method. Eighty two isolates could accumulate PHAs between 3.3 and 85.77% of their cell areas, which were observed under fluorescent microscope after cultivating on modified complex agar medium at 30°C for 48 h, then transferred to modified minimal medium with incubating at the same condition and duration as the complex medium. These PHAs-producing bacteria were identified by their morphological and physiological characteristics, and 16S ribosomal RNA gene sequence. From cell morphology, the bacteria could be divided into 4 groups: regular,

non-sporing Gram-positive rod, Gram-negative rod, endospore-forming Gram-positive rod, and Gram-positive coccus groups comprising 15, 3, 59, and 5 isolates; and accumulating PHAs in the range of 3.3-70.72%, 5.1-21.01%, 8.3-85.77%, and 51.9-74.99% of their cell areas, respectively. From physiological characterization, 8 genera: *Bacillus*, *Chryseobacterium*, *Enterobacter*, *Klebsiella*, *Listeria* (non-pathogenic species), *Micrococcus*, *Pseudomonas*, and *Staphylococcus* were classified. Then, representative isolates of these bacteria were selected for 16S ribosomal RNA gene analysis. Nucleotide sequences of 1,381-1,439 base pairs of the gene were obtained. When compared these sequences to sequences from GenBank database, it was found that 3 isolates of Gram-negative rods had 96.7, 99.1, and 99.5% similarity to *Pseudomonas aeruginosa* PAON 2, *Klebsiella oxytoca* SB 9, and *Enterobacter hormaechei* CIP 103441^{T}, respectively. Eight isolates of endospore-forming Gram-positive rods had 99.9, 99.2, 99.7, 98.0, 99.6, 99.2, 89.8, and 99.9% similarity to *Bacillus megaterium* ATCC 14581^{T}, *Bacillus cereus* ATCC 21281^{T}, *Bacillus* sp. NCCP-158^{T}, *B. cereus* ATCC 43881^{T}, *B. subtilis* KQC 85, *Bacillus* sp. IMT 21^{T}, *B. cereus* JS-33, and *Bacillus* sp. PBCC 10^{T}, respectively. And a representative strain of Gram-positive cocci had 99.3% similarity to *Staphylococcus cohnii* ATCC 29974^{T}. Two starch-hydrolysis strains isolated from cassava pulp sample, identified as belonging to *Bacillus megaterium*, and accumulating PHAs more that 80% of their cell areas, could have potential for biopolymer production.

School of Biology

Academic Year 2010

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Onuma Chansatein

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CHAPTER I

INTRODUCTION

1.1 Significance of the study

Polyhydroxyalkanoates (PHAs) are biopolymers synthesized by a wide range of microorganisms particularly bacteria (Kung *et al*., 2007; Sudesh and Abe, 2010). Bacteria accumulate PHAs under conditions of nutrient stress with an environmental excess of carbon. The PHAs typically are formed intracellular inclusions of 0.2-1 μm, and function primarily as carbon and energy reserves (Foster *et al*., 2005). Physical properties of PHAs are closed to thermoplastic polymers such as polypropylene (PP) derived from petrochemical industry and used in a wide variety of applications including packaging, textiles, automotive components, and polymer banknotes. PHAs are the only 100% biodegradable polymers, and have no toxic effect to living organisms. Microorganisms in nature are able to degrade PHAs by using their PHA hydrolases and PHA depolymerases (Jendrossek and Handrick, 2002; Choi, Kim, and Rhee, 2004; Sudesh and Abe, 2010). The activities of these enzymes may vary depending on the composition of the polymers and environmental conditions. Since PHAs have properties similar to synthetic polymers used for plastic industries, the major applications of PHAs are expected as the replacement for these petrochemical polymers, PHAs are also potentially useful for medical industries particularly heart valves, skin substitutes, and bone graft substitutes (Zinn, Witholt, and Eglia, 2001).

It has become clear that PHAs are synthesized in a wide range of bacteria

including Gram-negative, Gram-positive, and aerobic (cyanobacteria) and anaerobic (non-sulfur and sulfur purple bacteria) photosynthetic bacteria as well as some archaebacteria (such as the genus *Halobacterium*) (Lee, Choi, and Wong, 1999; Sudesh, Abe, and Doi, 2000). Methods for the identification of PHA-producing bacteria concern both conventional methods and molecular biology techniques. The accumulation of PHAs has been detected in about 300 bacterial species, particularly *Cupriavidus necator* (previously known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Alcaligenes latus*, *Pseudomonas oleovorans*, and *Pseudomonas putida* (Verlinden *et al*., 2007; Nubia *et al*., 2007). PHAs-producing bacteria can accumulate to levels reaching nearly 80% of the bacterial dry weight (Poirier, Erard, and MacDonald, 2002). Native PHA inclusions can be strained with straining dye such as Sudan black B, oxazine dye Nile blue A, and Nile red, and then observed under fluorescent microscope. The detection of PHA inclusion is further observed by transmission electron microscopy. Chemical analysis of the PHAs is often required to determine their monomeric compositions using gas chromatography (GC) and nuclear magnetic resonance (NMR) spectroscopy.

PHAs-producing bacteria have been reported to be found in various environments such as wastewater, industrial waste, municipal waste, soil, compost, hot spring water, fresh water and marine water (Sudesh, Abe, and Doi, 2000). The majority of PHAs-producing bacteria found in wastewater and soil are *Alcaligenes eutrophus*, *Pseudomonas aeruginosa* and *Methylobacterium* sp. (Yellore and Desai, 1998; Wu, Sheub, and Lee, 2003; Tsuge *et al*., 2004). Currently, there are a number of bacteria isolated from cassava pulp and kept in stock cultures in the Microbial Culture Collection and Application Research Unit at Suranaree University of Technology.

These bacteria have their basic characteristics close to PHAs-producing bacteria reported, and could be useful for PHA production. This research focused on investigation of the diversity of PHAs-producing bacteria isolated from cassava pulp samples, and physiological and 16S ribosomal RNA gene characterization of the bacteria to obtain data for further application of the strains.

1.2 Research objectives

1.2.1 To determine the diversity of PHAs-producing bacteria isolated from cassava pulp samples.

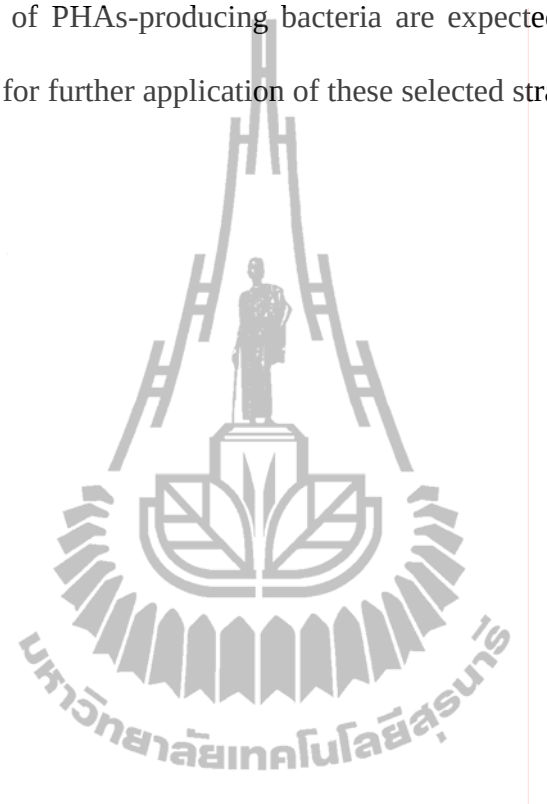
1.2.2 To physiologically and genetically characterize some selected strains of PHAs-producing bacteria to obtain data for further application of the strains for potential production of PHAs.

1.3 Scope and limitations of the study

At least 100 isolates of bacteria isolated from cassava pulp samples kept as stock cultures of the Microbial Culture Collection and Application Research Unit, Suranaree University of Technology, were examined for PHA accumulation within their cells. PHA-producing isolates were identified by their morphological and biochemical characteristics. The ribosomal gene characterizations of some selected strains were described. The diversity of PHAs-producing bacteria from the cassava pulp samples was concluded.

1.4 Expected results

The diversity of polyhydroxyalkanoates-producing bacteria isolated from cassava pulp, and data of physiological characteristics and 16S rRNA gene sequences of selected strains of PHAs-producing bacteria are expected to be achieved. These data will be useful for further application of these selected strains.

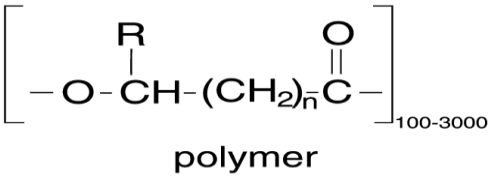


CHAPTER II

LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are biological polymers that are produced by a wide variety of bacteria as an intracellular storage material of carbon and energy reserves (Valentin *et al*., 1999; Tsuge *et al*., 2003; Kojima *et al*., 2004; Russell *et al*., 2006). The PHA polymers are biodegradable under aerobic and anaerobic conditions, and also have interesting material properties. Some are similar to those of the petrochemical-based polymers, polyethylene (PE) and polypropylene (PP), and exhibit good barrier properties against oxygen, water and oil (Valentin *et. al*., 1999). The biological polymers principally contain (R)-3 hydroxyalkanoic acids (HA) monomer units. The 3-HA units are all in the R configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase (PhaC). Poly(3-hydroxybutaric acid) (P(3HB)) containing repeat units of (R)-3HB, is the most well-known member of PHAs (Figure 2.1) and, are polymerized into high molecular weight polymers in the range of 200,000 to 3,000,000 daltons (Da), depending on microorganisms and their growth conditions (Sudesh, Abe, and Doi, 2000; Wu, Sheub, and Lee, 2003).



n = 1	R = hydrogen	poly(3-hydroxypropionate)	P(3HP)
	R = methyl	poly(3-hydroxybutyrate)	P(3HB)
	R = ethyl	poly(3-hydroxyvalerate)	P(3HV)
	R = propyl	poly(3-hydroxycaproate)	P(3HC)
	R = butyl	poly(3-hydroxyheptanoate)	P(3HH)
	R = pentyl	poly(3-hydroxyoctanoate)	P(3HO)
	R = hexyl	poly(3-hydroxynonanoate)	P(3HN)
	R = heptyl	poly(3-hydroxydecanoate)	P(3HD)
	R = octyl	poly(3-hydroxyundecanoate)	P(3HUD)
	R = nonyl	poly(3-hydroxydodecanoate)	P(3HDD)
n = 2	R = hydrogen	poly(4-hydroxybutyrate)	P(4HB)
n = 3	R = hydrogen	poly(5-hydroxyvalerate)	P(5HV)

Figure 2.1 Chemical structures of PHAs produced by bacteria.
Source: Wu *et al.* (2003).

PHAs have been broadly divided into three classes, (1) short-chain-length PHAs (SCL-PHAs) for polymers containing 3-hydroxyalkanoic acid monomers of three to five carbons, (2) medium-chain-length PHAs (MCL-PHAs) for polymers containing 3-hydroxyalkanoic acid monomers of six to 14 carbons, and (3) long-chain-length PHAs (LCL-PHAs) for polymers containing 3-hydroxyalkanoic acid monomers more than 14 carbons. SCL-PHAs have properties close to conventional plastics while MCL-PHAs are generally regarded as elastomers and rubbers (Poirier, Erard, and MacDonald, 2002).

2.1.1 Biosynthesis of PHAs

In the biosynthesis of PHAs, the polymerizing enzyme (PhaC) has been identified as the key enzyme which determines the type of PHA synthesized by bacteria (Sudesh, Abe, and Doi, 2000). Almost 40 PHA synthase structural genes from various Gram-positive and Gram-negative bacteria as well as cyanobacteria have

been cloned, and the nucleotide sequences from 30 genes have been identified. Three different types of PHA syntheses can be distinguished with respect to their substrate specificities and primary structures (Sudesh, Abe, and Doi, 2000). β-ketothiolase (PhaA) is a homotetrameric enzyme responsible for the reversible condensation of two molecules of acetyl-CoA to acetoacetyl-CoA and free CoASH, the first step in the P(3HB) biosynthesis pathway (Figure 2.2). NADPH-dependent acetoacetyl-CoA reductase (PhaB) is a homotetrameric enzyme responsible for the reduction of acetoacetyl-CoA to (D)-3-hydroxybutyryl-CoA, the second step of the P(3HB) biosynthesis pathway. PhaC, a key enzyme in PHA biosynthesis, polymerizes hydroxyacyl-CoA thioesters, which can be supplied from various metabolic pathways, into PHA. PhaP (phasin) is the predominant structural protein forming a layer via its association with PHA granule and determines the size and number of PHA granules. PhaR is a regulatory protein that binds an upstream region of the *pha*P gene to control the expression of phasin. PhaZ is a depolymerase enzyme which hydrolyzes PHA to a monomer (Kojima *et al*., 2004).

2.1.2 Biodegradability of PHAs in environment

PHAs are the only 100% biodegradable polymers. Microorganisms in nature are able to degrade PHAs by their PHA hydrolases and PHA depolymerases (Jendrossek and Handrick, 2002; Choi, Kim, and Rhee, 2004). PHAs are also completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage (Khanna and Srivastava, 2005). The activities of enzymes may vary and depend on the composition of the polymer and environmental conditions.

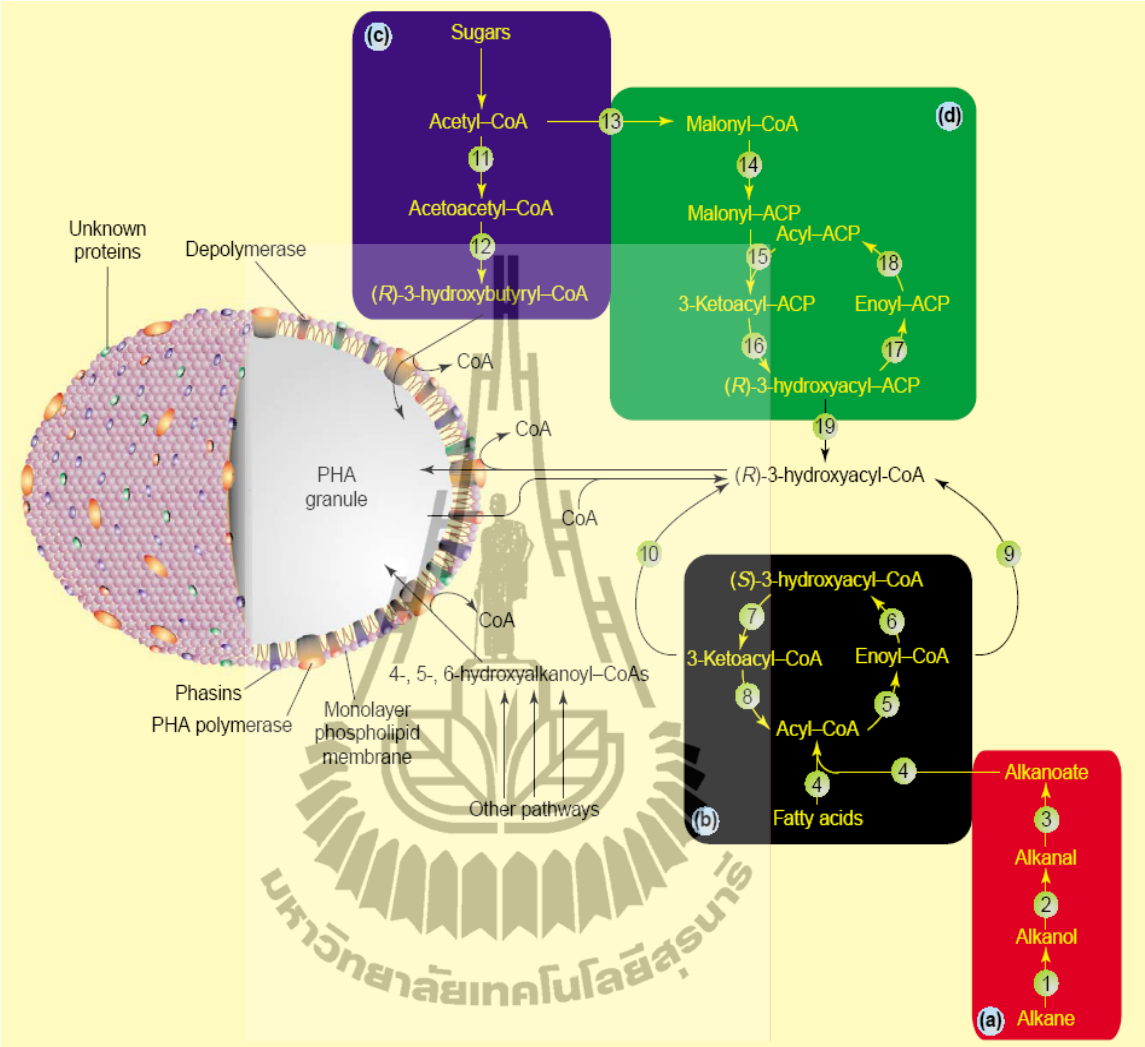


Figure 2.2 Structural organization of a PHA granule and metabolic interconnections between the different pathways involved in the biosynthesis of PHAs:

(a) Alkane oxidation pathway: (1) Alkane 1-monooxygenase, (2) alcohol dehydrogenase, (3) aldehyde dehydrogenase;

(b) Fatty-acid β-oxidation: (4) acyl-CoA ligase, (5) acyl-CoA dehydrogenase, (6) enoyl-CoA hydratase, (7) 3-hydroxyacyl-CoA dehydrogenase, (8) 3-ketothiolase, (9) (*R*) enoyl-CoA hydratase, (10) 3-ketoacyl-CoA reductase;

(c) Biosynthesis from carbohydrate: (11) β-ketothiolase, (12) NADPH-dependent acetoacetyl-CoA reductase;

(d) De novo fatty acid synthesis: (13) acetyl-CoA carboxylase, (14) ACP-malonyltransferase, (15) 3-ketoacyl-ACP synthase, (16) 3-ketoacyl-ACP reductase, (17) 3-hydroxyacyl-ACP reductase, (18) enoyl-ACP reductase, (19) 3-hydroxyacyl-ACP-CoA transacylase.

Source: Luengo *et al*. (2003).

For example, the degradation rate of a piece of PHB is typically in the order of a few months (in anaerobic sewage) to years (in seawater) (Madison and Huisman, 1999). UV light can accelerate the degradation of PHAs (Shangguan *et al*., 2006).

PHAs are environmental biocompatibility, and have no toxic effects in living organisms (Volova *et al*., 2003). Within mammals, the polymer is hydrolysed only slowly. After a 6-month period of implantation in mice, the mass loss was less than 1.6% (w/w) (Pouton and Akhtar, 1996).

2.1.3 Potential applications of PHAs

The major applications of PHAs are expected as the replacement for petrochemical polymers, PHAs are also potentially useful for medical applications, for example, medical grafting particularly heart valves, skin substitutes, and bone graft substitutes (Zinn, Witholt, and Eglia, 2001). Grabow *et al*. (2004) used poly(4-hydroxybutyrate) (P(4HB)) for graft substitutes. P(4HB) is PHA4400 with a molecular weight of 450,000 Da. A poly(3-hydroxybutyrate-co4-hydroxybutyrate) (P(3HB-co4HB)), is also known as PHA3444 with a composition of 82% 3HB and 18% 4HB having molecular weight of 310,000 Da. These polymers were used for test-tissue engineering heart valve for surgical treatment of heart valve diseases patients. Other potential application of PHAs in medicine described by Zinn, Witholt, and Eglia (2001) (Table 2.1).

The commodity plastics currently used for packaging and coating applications, can be replaced partially or entirely by PHAs (Verlinden *et al*., 2007). Bioplastic range of PHAs with 0-24% hydroxyvalerate has been produced in several countries particularly the United State of America (U.S.A.), United Kingdom (U.K.),

Austria, Canada, Italy and Germany (Table 2.2) (Salehizadeh and Van Loosdrecht, 2004).

Table 2.1 Potential applications of PHAs in medicine.

Type of application	Products
Wound management	Sutures, skin substitutes, nerve cuffs, surgical meshes, staples
Vascular system	Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts
Urology	Urological stents
Drug delivery	Micro- and nanospheres for anticancer therapy
Orthopaedy	Scaffolds for cartilage engineering, spinal cages, bone graft substitutes, meniscus regeneration, internal fixation devices
Dental treatment	Barrier material for guided tissue regeneration in periodontitis
Computer assisted tomography and ultrasound imaging	Contrast agents

Source: Zinn *et al.* (2001).

Table 2.2 Manufacturer and microorganisms used for the production of bioplastics.

Microorganism	Manufacturer
Alcaligenes eutrophus	ZENECA Bio Product, U.K.
Alcaligenes latus	Biotechnologische Forschungs gesellschaft, Austria
Recombinant *Escherichia coli*	Bioventures Alberta, Canada, Warner's Lambert, U.S.A.; Fertec, Novamont Novara, Italy; Biotech Emmerich, BASF, Ludwigshafen, Bayer Wolf Walsorde Leverkusen, Germany

Source: Salehizadeh and Van Loosdrecht (2004).

2.2 Microbial production of PHAs

A wide variety of microorganisms, particularly bacteria and fungi, can synthesize PHAs from a variety of substrates for each microorganism (Table 2.3). These microorganisms are capable of accumulating PHAs in large amounts, varying from 30 to 80% of their cellular dry weight (Kim and Lenz, 2001).

Table 2.3 Accumulation of PHAs in bacteria.

Genus	Group[a]	PHAs (%wt)	Substrate for PHA production
Acinetobacter	10	<1	Glucose
Aphanothece	CB	<1	NS
Aquaspirillum	6	ND	NS
Azospirillum	6	57	3-Hydroxybutyrate
Axobacter	7	73	Glucose
Bacillus	15	25	Glucose
Beggiatoa	2	57	Acetate
Beijerinckia	7	38	Glucose
Caulobacter	4	36	Glucose/glutamate/yeast extract
Chloroflexus	1	<1	Yeast extract/glycylglycine
Chlorogloea	CB	10	Acetate, carbondioxide
Chromatium	1	20	Acetate
Chromobacterium	8	37	Glucose/peptone
Clostridium	15	13	Trypton/peptone/glucose
Derxia	7	26	Glucose
Ectothiorhodospira	1	ND	NS
Escherichia[b]	8	ND	Prypton/glucose/yeast extract
Gampnosphaeria	CB	ND	ND
Haemophilus[b]	8	ND	Brain-heart-infusion
Halobacterium	13	38	Glucose
Hyphomicrobium	4	ND	Methanol, glucose
Lamprocystis	1	ND	NS
Lampropedia	10	ND	NS
Leptothrix	3	67	Pyruvate
Methylobacterium	7	47	Methanol
Methylocystis	ND	70	Methane
Methylosinus	7	25	Methane
Micrococcus	14	28	Pentone/trypton
Microcoieus	CB	<1	NS
Microcystis	CB	ND	ND
Moraxella	10	ND	NS
Mycoplana	17	ND	Methanol
Nitrobacta	12	ND	NS
Nitrococcus	12	ND	NS
Nocardia	17	14	Butane
Oceanospirillum	6	ND	NS
Paracoccus	10	ND	NS
Photobacterium	8	ND	NS
Pseudomonas	7	67	Methanol
Ralstronia	7	96	Glucose
Rhizobium	7	57	Methanol
Rhodobacter	1	60	Acetate
Rhodospirillum	1	47	Acetate
Sphaerotilus	3	45	Glucose/peptone
Spirillum	6	40	Lactate

Table 2.3 (Continued) Accumulation of PHAs in bacteria.

Genus	Group[a]	PHAs (%wt)	Substrate for PHA production
Acinetobacter	10	<1	Glucose
Spirulina	CB	6	Carbondioxide
Streptomyces	17	4	Glucose
Syntrophomonas	9	30	Crotonate
Thiobacillus	12	ND	Glucose
Thiocapse	1	ND	NS
Thiocystis	1	ND	NS
Thiodictyon	1	ND	NS
Thiopedia	1	ND	NS
Thiosphaera	1	ND	Acetone, carbondioxide
Vibrio	8	ND	NS
Xanthobacter	7	ND	NS
Zoogloea	7	ND	Yeast extract/casamino acids

[a]: Grouping according to Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994): 1, The spirochetes; 2, Aerobic/microaerophilic, motile, helical/vibrioid Gram-negative bacteria; 3, Non-motile (or rarely motile), Gram-negative curved bacteria; 4, Gram-negative, aerobic/microaerophilic rods and cocci; 6, Gram-negative, anaerobic, straight, curved, and helical rods; 7, Dissimilatory sulfate- or sulfur-reducing bacteria; 8, Anaerobic Gram-negative cocci; 9, The rickettsias and chlamydias; 10, Anoxygenic phototrophic bacteria; 12, Aerobic chemolithotrophic bacteria and associated organisms; 13, Budding and/or appendaged bacteria; 14, Sheathed bacteria; 15, Nonphotosynthetic, nonfruiting gliding bacteria; and 17, Gram-positive cocci.

[b]: PHB found in cell membranes

%wt: Percentage of cellular dry weight

CB: Cyanobacteria

ND: Not detected

NS: Not selected

Source: Kim and Lenz (2001).

2.3 Bacteria as the potential PHAs-producing organism

Several bacterial species were evaluated as PHAs potential production organisms (Madison and Huisman, 1999). The storage polymers are synthesized not only in Gram-negative bacteria but also in a wide range of Gram-positive bacteria, aerobic (cyanobacteria) and anaerobic (non-sulfur and sulfur purple bacteria) photosynthetic bacteria, as well as in some archaebacteria (Lee, Choi, and Wong, 1999; Sudesh, Abe, and Doi, 2000), which are about 300 bacterial species such as

Cupriavidus necator (previously known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *A. latus*, *Pseudomonas oleovorans*, *P. putida*, and *Azotobacter vinelandii* (Verlinden *et al*., 2007; Nubia *et al*., 2007). PHAs typically form intracellular inclusions of 0.2-1 µm diameters and can accumulate to levels reaching nearly 80% of the bacterial dry weight (Poirier, Erard, and MacDonald, 2002).

2.4 Accumulation of PHAs in bacterial cells

PHAs are accumulated as water-insoluble granules in the bacterial cytoplasm. Bacteria produce PHA inclusions under nutrient stress where a carbon source is provided in excess quantity or any other essential nutrient is limited (Khanna and Srivastava, 2005; Zhao *et al*., 2006). PHAs typically form intracellular inclusions of 0.2-1 µm diameters, and can accumulate to levels reaching nearly 80% of the bacterial dry weight (Valentin *et al*., 1999; Poirier, Erard, and MacDonald, 2002). When thin sections of PHAs-containing bacteria are observed by transmission electron microscopy, the PHA inclusions appear as electron-dense bodies (Figure 2.3). Native PHA inclusions can be stained with Sudan black B or fluorescent stains, Nile blue and Nile red, indicating that they are of a lipid nature (Sudesh, Abe, and Doi, 2000; Zinn, Witholt, and Eglia, 2001). This accumulation in granules separates the polymers from the cell lumen and, consequently, the osmotic pressure of the cell is not changed extensively. The number of granules per cell seems to be strain specific. For example, *Ralstonia eutropha* has between 8 and 12 granules of variable size, whereas *Pseudomonas oleovorans* is estimated to have about 1 or 2 large granules (Zinn, Witholt, and Eglia, 2001). PHA inclusion is composed of protein and lipid. The core consists of PHA polymers that are covered by a lipid monolayer with integrated

proteins. The integrated proteins consist of PHA polymerase, PHA depolymerase, structural protein (phasin), and proteins of unknown function. The surface of *in vivo* PHAs inclusions is a dynamic boundary region where the proteins involved in the biosynthesis (PHA synthase) and degradation and/or mobilization (intracellular PHA depolymerase) of PHAs are located. Besides these, other proteins (phasins) thought to be involved in the formation and stabilization of PHA inclusions has also been identified on the surface. The lipid monolayer points with the hydrophobic side to the core (Zinn, Witholt, and Eglia, 2001).

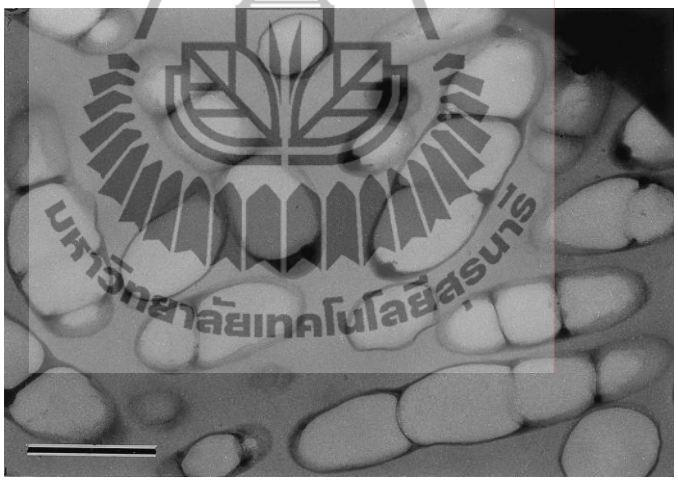


Figure 2.3 Transmission electron micrograph of thin sections of recombinant *Ralstonia eutropha*, PHAs, Bar represents 0.5 µm.
Source: Sudesh, Abe, and Doi, (2000).

The physical state of poly(3-hydroxybutyrate) (P(3HB)) inclusions is able to demonstrate using solution-state nuclear magnetic resonance (NMR) techniques. The bulk of P(3HB) *in vivo* is not crystalline. It is actually a mobile amorphous polymer (Madison and Huisman, 1999; Sudesh, Abe, and Doi, 2000). This explains why mild treatments such as centrifugation lead to the rapid and irreversible loss of its degradability by the intracellular depolymerizing enzyme (Sudesh, Abe, and Doi,

2000). However, after extraction from the cell with organic solvents, P(3HB) becomes highly crystalline, and in this state, it is a stiff but brittle material. Because of its brittleness, P(3HB) is not very stress resistant. Also, the relatively high melting temperature (Tm) of P(3HB) is around 170°C close to the temperature where this polymer decomposes thermally, and thus limits the ability to process the homopolymer. Biotechnological developments are therefore aimed at making PHAs that are easier to process. The incorporation of poly(3-hydroxyvalerate) (P(3HV)) into the P(3HB) resulted in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-3HV)) copolymer that is less stiff and brittle than P(3HB). It can be used to prepare films with excellent water and gas barrier properties reminiscent of polypropylene, and can be processed at a lower temperature while retaining most of other excellent mechanical properties of P(3HB). When an elastic or plastic polymeric material is cooled below its glass transition temperature (Tg), there may no longer be able to dissipate energy by dimensional changes, and will tend to exhibit brittle fracture (Sudesh, Abe, and Doi, 2000). Properties of PHAs and polypropylene (PP) were shown in Table 2.4.

Table 2.4 Properties of PHAs and polypropylene.

Parameter	Value for				
	P(3HB)	P(3HB-3HV)	P(3HB-4HB)	P(3HO-3HH)	PP
Tm (°C)	177	145	150	61	176
Tg (°C)	2	-1	-7	-36	-10
Crystallinity (%)	70	56	45	30	60
Extension to break	5	50	444	300	400

P(3HB), Poly(3-hydroxybutyrate)
P(3HB-3HV), Poly(3-hydroxybutyrate)-poly(3-hydroxyvalerrate)
P(3HB-4HB), Poly(3-hydroxybutyrate)-poly(4-hydroxybutyrate)
P(3HO-3HH), Poly(3-hydroxyoctanoate)-poly(3-hydroxyheptanoate)
PP, Polypropylene
Source: Madison and Huisman (1999).

2.5 Detection of PHAs-producing bacteria

There are several methods for the detection of PHAs-producing bacteria. Conventional culture methods and genetic methods are wildly used for the detection of these bacteria.

Conventional culture methods for the detection of PHAs-producing bacteria are based on directly using cultural medium and staining with lipophilic dye such as Sudan black or Nile blue. Although highly sensitive, these reagents also act with other lipid inclusions and thus are not specific (Ciesielski *et al*., 2006).

For genetic methods, four major classes of PHA biosynthesis loci have been characterized (Ciesielski *et al*., 2006). In the first class, the gene for *pha*C, *pha*A, and *pha*B constitute the *pha*CAB operon. It has been found in *Ralstonia eutropha*. The second class of the PHA synthesis system contains two synthase genes (*pha*C1 and *pha*C2) disjoined by the gene coding for the *pha*Z in *Pseudomonas* sp. In the third class, the synthase enzyme consists of two subunits that are encoded by *pha*E and *pha*C genes, have also been reported in *Chromatium vinosum* and *Synechocystis* sp. And fourth class is the *pha*C1ZC2 operon, which is well characterized, and DNA sequences of *pha*C1/*pha*Z/*pha*C2 genes of *Pseudomonas* sp. studied are homologous. Strategies based on PHA synthase gene detection, include homologous and heterologous gene probes, short consensus oligonucleotides hybridization or polymerase chain reaction (PCR) techniques could be more reliable for the detection of PHAs-producing bacteria (Ciesielski *et al*., 2006).

2.6 Methods for the detection of PHA granules in bacterial cells

PHA granules in bacterial cells are detected base on cultural medium and staining with lipophilic dyes (Ostle and Holt, 1982; Lee *et al*., 1994). Purified isolates will be screened for PHA accumulation. The isolates will first grow in a nutrient-rich medium in order to produce large amount of cells. After incubation, the cells will be harvested and washed. Bacteria will be transferred to minimal medium and incubation (Lee *et al*., 1994). A loopful of bacteria culture will be smeared on a glass slide, heat-fixed and stained with Nile blue A to detect the presence of intracellular PHA granules under the fluorescent microscope, which provides an excitation wave length of approximately 460 nm (Ostle and Holt, 1982).

2.7 Chemical characterization of PHAs from bacterial cells

Chemical analysis is often required to determine monomeric compositions of PHAs from bacterial cells. Gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy analyses are widely used for the detection of PHAs.

Gas chromatography is a chromatographic separation method, which the sample is dissolved in gas mobile phase, the sample is vaporized and injected into head of a chromatographic column. GC has also been applied to separate PHA monomeric compositions and determine the content of PHAs from bacteria particularly PHA monomer composition from *Pseudomonas* sp. which was determined using GC-8000 series gas chromatograph (Tobin and O'Connor, 2005), and GC was also used for analysis of intracellular PHA content and PHA composition from *P. mendocina* (Zheng *et al*., 2005).

Gas chromatography-mass spectroscopy is a type of GC. GC separates the components of a mixture, and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals. As the individual compounds elute from the GC column, they enter the electron ionization detector. It is bombarded with a stream of electrons causing them to break apart into fragments. These fragments can be large or small pieces of the original molecules. This instrument use for the detection of PHAs from bacterial cells such as *P. oleovorans* which was extracted from freeze-dried cell biomass and analyzed by GC-MS (Holden *et al*., 2004; Foster *et al*., 2005). Methyl ester of PHA monomers from *P. putida* were analyzed by GC-MS to identify the composition of the polymer (Takagi *et al*., 2004); and *P. guezennei* was isolated from marine microbial mats in France. This bacterium produced a novel MCL-PHAs, mainly composed of 3-hydroxydecanaote (64% moles) and 3-hydroxyoctanoate (24% moles) which is detected by GC-MS (Takagi *et al*., 2004).

Nuclear magnetic resonance is a technique which exploits the magnetic properties of certain nuclei. This method use infrared spectroscopy to identify functional groups, analysis of a NMR spectrum provides information on the number and type of chemical entities in a molecule. NMR was used for the detection of PHAs from bacterial cells particularly PHAs production from *P. oleovorans*, which was used ^{1}H and ^{13}C NMR for identification (Gross *et al*., 1989). ^{31}P NMR was used for the detection of PHAs from *R. eutropha* (Madden *et al*., 1999). PHAs from *Bacillus* sp. INTO05 was determined by ^{1}H-decoupled NMR (Tajima *et al*., 2003); and ^{1}H and

^{13}C NMR was also reported for the detection of PHAs from *Cupriavidus necator* (Pederson, McChalicher, and Srienc, 2006).

2.8 Sources of PHAs-producing bacteria

PHAs-producing bacteria have been reported to be found in various environments such as wastewater, industrial waste, municipal waste, soil, compost, water particularly hot spring, fresh, and marine water (Sudesh, Abe, and Doi, 2000).

2.8.1 Water

Bacteria isolated from fresh water and grown on heterotrophic plate count (HPC) represent the aerobic and facultative anaerobic bacteria. This group includes Gram-negative bacteria belonging to the following genera: *Pseudomonas*, *Aeromonas*, *Klebsiella*, *Flavobacterium*, *Enterobacter*, *Citrobacter*, *Serratia*, *Acenetobacter*, *Proteus*, *Alcaligenes*, *Enterobacter*, and *Moraxella* (Bitton, 1994). Bacteria can accumulate PHAs in intracellular from organic acids under nitrogen limited conditions, or under conditions of a high carbon and nitrogen ratio in water and wastewater (Hassan *et al*., 1997).

2.8.1.1 Wastewater and activated sludge

Lee, Rang, and Choi, (1995) screened a large number of bacterial isolates from activated sludge for their ability to synthesize P(3HBco-3HV). *Agrobacterium* sp. SH-1 and GW-014, could synthesize P(3HBco-3HV) with 3HV monomer mole percent in the range from 1.3 to 11.4 from single carbon substrate, such as glucose, xylose or sucrose. Alias and Tan (2005) isolated two isolates FLP1 and FLP2, that could utilize palm olein in palm oil mill effluent (POME) for growth

and production of the homopolyester, P(3-HB). FLP1, a Gram-negative bacterium, has 80% similarity to *Burkholderia cepacia*. When grown with propionate or valerate, FLP1 produced a copolyester, P(3-HB-co-3-HV).

Medium chain length (MCL)-PHAs-producing bacteria have been reported from activated sludge (Ciesielski *et al*., 2006). Three strains were selected for 16S rDNA sequence analysis. They belonged to *Pseudomonas* species, *P. fluorescens*, and *P. aeruginosa* groups, and another strain was characterized as *Comamonas testosteroni*.

A group of uncultured Gamma-proteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants (Kong *et al*., 2006). PHA granules after Nile blue staining were found in most of the Gamma-proteobacteria.

Ecophysiology of abundant denitrifying bacteria in activated sludge was also reported (Thomsen, Kong, and Nielsen, 2007). The most abundant potential denitrifiers were related to genera *Aquaspirillum*, *Azoarcus*, *Thauera* and *Rhodocyclus*, all within the Beta-proteobacteria.

2.8.1.2 Local pond water

Methylobacterium sp. ZP24 is the bacterial strain isolated from a local pond, able to grow in a medium containing 12 g/L lactose as a sole source of carbon (Yellore and Desai, 1998). It was give 5.25 g/L biomass yield and PHB up to 59% of its dry weight in 40 h. PHAs-producing bacteria were isolated from soil and pond water samples in Bangkok, Thailand (Lorrungruang *et al*., 2006). The purple non-sulfur photosynthetic bacterium belonging to the genus *Rhodobacter* was

reported. A strain identified as *Rhodobacter sphaeroides* showed the highest PHA production (3.5 g/L PHAs with 60% PHAs content). Its productivity was 2-3 times higher than those of other photosynthetic bacteria.

2.8.1.3 Saline water

Aerobic phototrophic bacteria population in the Mahoney saline lake located near Penticton in the dry region of south central British Columbia, were isolated (Yukova *et al*., 2002). The pink-colored strain ML10 was found to be motile and formed ovoid rods or elongated cells of irregular shape. Massive formation of presumably PHAs granules was a specific feature of this strain. Based upon partial 16S rDNA sequences, all studied isolates belong to the Alpha subclass of proteobacteria. With similarity values of isolates ranging between 97 and 100% are closely related to species of genera *Agrobacterium*, *Erythrobacter*, *Erythromicrobium*, *Porphyrobacter*, and *Sphingomonas*. A new PHA-producing strain designated as RA26 which was isolated from a 'kopara' mat of the Rangiroa atoll in France (Simon-Colin *et al*., 2007). Phylogenetic analyses using the ARB program showed that strain belonged to the Gamma subdivision of the phylum Proteobacteria and that it was closely related to *P. aeruginosa*. During the batch fermentation of strain RA26, the production of PHA began after 34 h in glucose-enriched medium and stopped after 3 days. Under these conditions, the yield of PHA produced was near 250 mg/L, with granules filling about 80-90% of the cellular volume. Diversity and activity of polyhydroxyalkanoate-producing and -degrading bacteria and their close partnership with cyanobacteria in an estuarine and a hypersaline microbial mat was studied by Villanueva, Del Campo, and Guerrero

(2010). Most of the polyhydroxyalkanoate-producing isolates obtained from an estuarine mat belonged to *Halomonas* and *Labrenzia* genera, while species of *Sphingomonas* and *Bacillus* were more prevalent in the hypersaline mat.

2.8.2 Soil

PHAs-producing bacteria had been found in soil both from agricultural fields and industrial waste drainage sites, where environment in nutrient stress condition.

2.8.2.1 Agricultural field soil

PHAs-producing bacteria are isolated from agricultural soil such as sugarcane and soybean field. Two isolates (2G57 and C14) were isolated from soil and rhizosphere samples from sugarcane growing farms located in Colombia (Nubia *et al*., 2007). At fermenter level, the isolates presented PHA productivity to 0.059 and 0.0671 g/L/h for 2G57 and C14, respectively.

PHAs-producing bacteria have been isolated from samples taken from soybean field, Crystal Lake, and Lake Murphysboro sediments in South Illinois (Full, Jung, and Madigan, 2006). The isolates were identified as belonging to *Bacillus* species. A strain produced PHAs reaches 90% of cell dry mass by using raffinose. PHAs-producing bacteria from an agricultural field, industrial waste drainage sites, oil contaminated field, and hot spring areas were also reported (Kung *et al*., 2007). Bacteria isolated from these environments were screened for PHA production. More than a hundred strains produce and accumulate significant amount of PHAs. The strains belong to genera *Pseudomonas, Aeromonas, Bacillus, Enterobacter, Acinetobacter*, and *Exiguobacterium*.

2.8.2.2 Contaminated industrial waste soil

PHAs-producing bacteria have been reported to be found in industrial waste drainage site soil such as oil-contaminated and gas field soil. A strain of *Pseudomonas stutzeri* isolated from oil-contaminated soil was found to grow well in glucose and soybean oil as a sole carbon source, respectively (He *et al*., 1998). The bacteria produced PHAs containing MCL-monomers of hydroxyalkanoates ranging from C6 to C14 synthesized up to 52% of cell dry weight, when grown on glucose mineral media and produced up to 63% in mineral media containing 10 g/L soybean oil. *Pseudomonas mendocina*, a proteobacterium from oil-contaminated soil samples in Northern China was also reported to produce PHAs (Zheng *et al*., 2005). Gram-positive bacteria accumulating PHAs and isolated from gas field soil, belonged to genera *Bacillus* and *Ralstonia* (Tajima *et al*., 2003). PHA productivities of these strains were higher than *Bacillus megaterium* and *Ralstonia eutropha* growing at 37-45°C.

2.9 Characterization of bacteria

2.9.1 Morphological characterization

One of the properties of major importance in classifying bacteria is morphology of the individual bacterial cell particularly cell shape (rods or bacilli, cocci and spiral shape), cell arrangement (singly, or in chains, clusters, or regular packets), flagella arrangement, and the reaction after staining by the method of Gram. Gram-stained bacterium reveals another significant morphological character. It tells whether a bacterium does or does not possess an outer membrane and whether it has a thick or thin peptidoglycan wall. This simple test distinguishes two major divisions of

bacteria, Gram-negative and Gram-positive (Pattison *et al*., 1995; Ingraham and Ingraham, 2000).

2.9.2 Physiological characterization

Physiological characterization used to classify bacteria is based on conditions that support their growth including a supply of suitable nutrient, a source of energy, an appropriate temperature, an appropriate pH, and appropriate level (or the absence) of oxygen (Singleton, 2004). Carbon sources that support growth are a particularly useful set of characters because most bacteria can use so many different carbon sources. Bacteria are divided into thermophilic, mesophilic and psychrophilic bacteria depending on their appropriate temperature for growth. Most bacteria grow best at or near pH 7.0 (neutral), and the majority cannot grow under strongly acidic or strongly alkaline. However, some of bacterial species are tolerated to those conditions. They are acidophiles or alkalophiles. An appropriate level of oxygen has affected to bacterial growth. Bacteria are sensitive to oxygen classified as obligate anaerobes, tolerance of it is aerobes or requirement for it is strict aerobes. Biochemical characterizations of bacteria are also useful characters for bacterial metabolisms.

2.9.3 Genetic characterization

Genetic characterization of bacteria has been acquired through the application of techniques based on the complementary nature of the double-helix structure of deoxyribonucleic acid (DNA) (Ogunseitan, 2005). The techniques related with percentage of guanine+cytosine (% G+C) and DNA hybridization which are

highly indirect methods of evaluating bacterial genomes. The mole % G+C expresses the percentage of total base pairs in DNA that are G-C, as opposed to adenine-thymine (A-T). Higher % G+C means a higher DNA melting point and density. Closely related bacteria have similar % G+C values. DNA hybridization involves heating DNA melts it, allowing the two strands of the double helix to separate, DNA is cooled, and the two single strands anneal. When single strands of DNA from different sources are mixed, they anneal in regions where the sequences are the same or similar, creating a hybrid molecule. Therefore, the extent of annealing is a quantitative index of the similarity of base sequences in the DNA from the two sources.

Polymerase chain reaction (PCR) is a method for copying specific sequences of nucleotides in DNA or in a modified form of the process in RNA and repeated replication of a given sequence of nucleotides (the PCR product usually less than 2 kb long) forms millions of copies within hours (Singleton, 2004). This method is a powerful technique which has been widely used for the identification of bacteria. For example, PCR techniques are used for the detection of PHAs-producing bacteria show in Table 2.5.

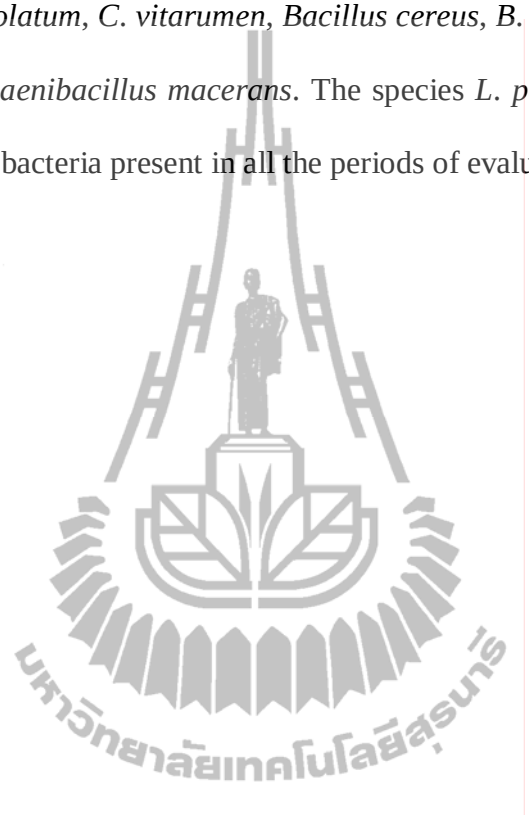
Table 2.5 PCR primers for the detection of PHAs-producing bacteria.

Primer name and sequence	PCR product (bp)	Target gene	Specific bacteria and reference
B1F: (5'AACTCCTGGGCTTGAAGACA 3')		*pha*C gene	*Bacillus* sp.
B1R: (5'TCGCAATATGATCACGGCTA 3')	590		Shamala *et al.*
B2R: (5'ACGGTCCACCAACGTTACAT 3')	380		(2003)
I-179L: (5'ACAGATCAACAAGTTCTACATCTTCGAC 3')	540	*pha*C1	PHAs-
I-179R: (5'GGTGTTGTCGTTGTTCCAGTAGAGGATGTC 3')	540	and	producing
16SR: (5'TACCTTGTTACGACTTCACCC CA 3')	1124	*pha*C2	bacteria
8F: (5'GTGCTGCAGAGAGTTTGATCCTGGCTCAG 3')	234	genes	Ciesielski *et al.*
536R: (5'CACGGATCCGTATTACCGCGGCTGCTG 3')	530		(2006)

2.10 Bacteria found in cassava products and cassava pulp

Cassava or manioc (*Manihot esculenta* Crantz), a perennial shrub, currently is the sixth world food crop for more than 500 million people in tropical and sub-tropical Africa, Asia and Latin America (El-Sharkawy, 2004). Cassava pulp is the solid waste produced as a consequence of starch production. This pulp contains a high starch content (50-60% dry basis), which yields around 1.5-1.8 million metric tons annually in Thailand. The main application for the large quantities of waste material produced each year, after drying, is as animal feed or fertilizer (Sriroth *et al*., 2000). Coulin *et al*. (2006) characterized the microflora of attie´ke´ (a fermented cassava root or pulp), during traditional small-scale preparation in the South of Côte d'Ivoire (Abidjan region). Bacteria isolated from cassava undergoing fermentation for production of attie´ke´ were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc citreum, Streptococcus constellatus, S. alactolyticus, Enterococcus faecalis, Lactobacillus buchneri, L. brevis, L. bifermentans, L. fermentum, L. confuses, L. delbrueckii* subsp. *delbrueckii, L. salivarius, L. amylovorus, L. jensenii*, and *L. plantarum*. Fagbemi and Ijah (2006) monitored the bacterial population and some biochemical changes during the production of fufu (a cassava creamy white fermented paste). Various bacteria (*Bacillus, Staphylococcus, Klebsiella, Escherichia, Streptococcus, Lactobacillus, Leuconostoc, Corynebacterium*) were found to be associated with the fermentation process. Almeida, Rachid, and Schwan (2007) isolated bacteria from 'fermented cassava-cauim' beverage at Mato Grosso, Brazil. A total of 355 bacteria were isolated and identified. All the isolates were grouped into Gram-negative (3.5%), Gram-positive non-sporulating (78%) and Gram-positive sporulating bacteria (18.5%). Lactic acid

bacteria increased from the beginning of fermentation and became the dominant microorganisms throughout the cassava fermentation. Species of bacteria were varied and they were found to be *Lactobacillus pentosus, L. plantarum, Corynebacterium xerosis, C. amylocolatum, C. vitarumen, Bacillus cereus, B. licheniformis, B. pumilus, B. circulans* and *Paenibacillus macerans*. The species *L. pentosus* and *L. plantarum* were the dominant bacteria present in all the periods of evaluation of the samples.



CHAPTER III

MATERIALS AND METHODS

3.1 Cultivation media, chemicals, and reagents for microbiological analysis

3.1.1 Media for isolation of PHAs-producing bacteria

The microbiological media preparations for isolation of PHAs-producing bacteria isolated from cassava pulp samples are given in Appendix A 1. Five difference agars including carboxymethylcellulose (CMC) agar, thermo-carboxymethylcellulose (TCMC) agar, plate count agar (PCA), trypticase soy agar (TSA), and starch agar (SA) (Appendix A 1.3-1.7, respectively) were used for the isolation. The media were described, according to Atlas (1997). Complex and minimal medium were developed and also described in Appendix A 1. Modified complex (Appendix A 1.1), and minimal medium (Appendix A 1.2) were developed base on potential references using for screening and culturing of PHAs-producing bacteria isolated from cassava pulp. Media for physiological identification of PHAs-producing bacteria were composed of triple sugar iron (TSI) medium, lysine indole motile (LIM) medium, methyl red Voges-Proskauer (MR-VP) medium, Simmons citrate agar, nitrate reduction broth, oxidation-fermentation medium, lauryl sulfate tryptose (LST) broth, motility test medium, nutrient gelatin, phenol red broth, starch hydrolysis agar, tributyrin agar, and skim milk agar (Appendix A 1.9-1.21, respectively).

3.1.2 Chemicals and reagents for physiological identification of PHAs-producing bacteria

Chemicals and reagents used for identification of the bacterial isolates based on conventional method were 1% solution of dimethyl-p-phenylenediamine dihydrochloride, Gram staining reagents, 3% hydrogen peroxide, Kovács' reagent, methyl red test reagent, nitrate test reagents, and Voges-Proskauer reagents (Appendix A 2.1-2.7, respectively). The preparations of these reagents were according to Beishier, 1991, and Smibert, and Krieg, 1994.

3.1.3 Culture media and chemicals for API identification system (bioMérieux, Inc., France)

Biochemical characterizations of PHAs-producing bacteria isolated from cassava pulp were proved by API identification system (bioMérieux, Inc., France). Cultural media and chemicals were trypticase soy broth (TSB), trypticase soy agar (TSA), standard McFarland, and sterile emulsion oil. All of these media and chemicals were given by sections 1 and 3 in Appendix A.

3.1.4 Reagents for 16S rRNA gene

The reagent preparations for nucleic acids analysis are given in Appendix A 4. Reagents used for DNA extraction were STE buffer, TE buffer, and Tris-saturated phenol (pH 8.0). Reagents used for amplification of 16S rDNA were PCR buffer without $MgCl_2$ (100 mM tris-HCl, pH 9.1; 500 mM KCl and Triton^{TM}X-100), $MgCl_2$ solution (25 mM $MgCl_2$ in sterile water), dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), primers, and *Taq* DNA polymerase (Vivantis,

VIVANTIS TECHNOLOGIES SDN BHD**,** Malaysia) The reagents used for sequencing were prepared as described in the manual of ABI377 Autometed DNA sequencer (Perkin Elmer, Applied Biosystems, Inc., Foster, CA, U.S.A.). The BigDye Terminator Ready Reaction kit (Perkin Elmer, Applied Biosystems Inc., U.S.A.) was used for the nucleotide sequencing reaction.

3.1.5 Chemicals and reagents for scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses

The chemicals and reagents used for electron microscopy techniques were composing of 5% glutaraldehyde, 1% osmiumtetoxide, 0.2 M phosphate buffer, 4 and 5% uranyl acetate, acetone series (20, 40, 60, 80, and 100%), 0.4% lead citrate staining, and Epon polymer. Preparation of these chemical were described on section 5 in Appendix A.

3.2 Instrumentation

Instruments required for the selection, detection and characterization of PHAs-producing bacteria were located in the Instrument Buildings of the Centre for Scientific and Technological Equipment at Suranaree University of Technology, Nakhon Ratchasima, Thailand. The instruments required for DNA sequencing were located at the Biotechnology Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperative, Pathum Thani, Thailand.

3.3 Selection of PHAs-producing bacteria

3.3.1 Collection of bacterial isolates

Bacterial isolates were obtained from the Microbial Culture Collection and Applications Research Unit, Suranaree University of Technology, and isolated from cassava pulp samples.

3.3.2 Bacterial isolation from cassava pulp samples

Cassava samples used for isolation of PHAs-producing bacteria were collected from modified cassava starch industrial factories, and sun drying field for cassava pulp in Northeastern Thailand. Twenty five grams of cassava pulp samples were mixed with 225 mL of phosphate buffer pH 7.2 (Appendix A 2.10) to make the 1:10 dilution. The homogeneous serial dilutions of $1{:}10^2$, $1{:}10^3$, $1{:}10^4$, $1{:}10^5$, $1{:}10^6$, $1{:}10^7$ and $1{:}10^8$ were prepared, and each of dilution was poured on five difference agars including carboxymethylcellulose (CMC) agar (Appendix A 1.3), thermo-carboxymethylcellulose (TCMC) agar (Appendix A 1.4), plate count agar (PCA) (Appendix A 1.5), trypticase soy agar (TSA) (Appendix A 1.6), and starch agar (SA) (Appendix A 1.7), and incubating at 35°C for 48 h. Difference size, color, edge, surface, whole colony, elevation, and diameter of colony were selected for PHA detection in their cells. The selected isolates were re-streaked on the original agar medium for bacterial purification. Purified isolates were kept in 5% skim milk (final concentration) at -80°C as stock culture and using for the detection of PHAs-producing bacteria.

3.3.3 Development of cultivation media for PHA accumulation in bacterial cells

Bacterial isolates from stock culture were re-streaked on TSA for pure culture preparation. The isolates characterized by morphological characteristics according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Then, the isolates were arranged by groups. Appropriate media for the detection of PHAs-producing bacteria were modified for each group, based on methods which were described by Lee *et al*. (1994); He *et al*. (1998); Kim *et al*. (2000); Yu *et al*. (2002); Kojima *et al*. (2004); Takagi *et al*. (2004); Foster *et al*. (2005); Zheng *et al*. (2005); Full *et al*. (2006); Berlanga *et al*. (2006); Ciesielski *et al*. (2007); and Halet *et al*. (2007). Selections media, twenty bacterial isolates from cassava pulp were tested for their growth in the investigated media. The bacterial isolates were grown in complex medium according to references (Lee *et al*., 1994; Fukui *et al*., 1998; Yu *et al*., 2002; Tajima *et al*., 2003; and Kung *et al*., 2007) and modified medium for this study. The flasks of cultures were incubated at 100 rpm 30°C for 24 h in an orbital incubator shaker (Heto Maxi-Shark, Heto-Holten, Gydevang, Denmark). Every 2 h of incubation, each sample was taken 2 mL from the flask. One milliliter of sample was diluted and spreaded on TSA plates. Determination of cells growth rate was used spectrophotometer (SHIMADZU RF-1501, SHIMADZU, Japan) at wavelength 600 nm. Optical density of the determination and bacterial growth on the plates were used for the selection of appropriated complex medium. Selection of minimal medium, high content of PHA accumulation in bacterial cells after cultured on minimal medium and incubated at 30°C for 48 h was selected for PHAs-producing bacterial cultivation.

3.3.4 Screening of PHAs-producing bacteria

Pure cultures isolated from cassava pulp were determined for PHAs accumulation in their cells. Modified complex and minimal media were developed as previous described (section 3.3.3). The purified isolates were cultured on complex, and then transferred to minimal cultivation medium which was supplemented with 0.5 mg/L Nile red (dissolved in 1 mL of dimethylsulfoxide) (Berlanga *et al*., 2006). Then, incubated at 30°C for 48 h. Development of pink colonies under UV light after cultured on minimal medium were selected for PHAs-producing bacteria analysis. *Alcaligenes eutrophus* TISTR 1095 and *Escherichia coli* TISTR 527 were used for positive and negative controls of PHAs-producing strains.

3.4 Detection of PHA accumulation in bacterial cells

3.4.1 Fluorescent microscopy for the detection of PHA accumulation in bacterial cells

Developments of pink colonies under UV light after cultured on minimal medium were selected for PHA accumulation in bacterial cells analysis. The isolates were detected for the presence of PHA accumulation by the methods according to Ostle and Holt (1982); Lee *et al*. (1994); and Rodtong *et al*. (2008). Pure cultures of bacteria were culturing on modified complex medium incubating at 30°C for 48 h, then transferred to minimal medium and incubating at the same condition. Heat-fixed smears of bacterial cells were stained with 1% Nile blue A solution (Appendix A 2.8), and observed under fluorescence microscope (Fluorescence microscope; Olympus Model BX51TRF, Olympus Optical Co., Ltd., Japan) at excitation wave length of 650 nm. *Alcaligenes eutrophus* TISTR 1095 was used as the positive control of PHAs-

producing strains. Percentage of PHA accumulation in bacterial cells was measured by Immage-Pro Plus Version 6.0.0.Z60 (Media Cybematics, Inc., Japan) from density of bright orange color when observed under fluorescent detector from fluorescent microscope. Bacterial isolates accumulating high content of PHAs were selected for electron microscopy detection.

3.4.2 Electron microscopy for the detection of PHA accumulation in bacterial cells

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used for detecting PHAs in bacterial cells. Fixative solution was used composing of 5% glutaraldehyde, 1% Osmiumtetoxide, 0.2 M phosphate buffer (Appendix A 5.1) in the ratio of 1:1:1. The mixture was kept at 4°C until use. Colonies of selected bacteria were transferred to Eppendorf tube (1.5 mL) containing 1 mL of fresh fixative solution and incubating at 4°C for 2 h. The sample preparations were made for duplication of TEM and SEM techniques. After 2 h of fixation, the bacterial cells were spun down at 8,000 rpm for 10 min using a micro centrifuge. The supernatant was removed, and an additional 0.1 M of phosphate buffer 1 mL was added to wash the cell pellet for 10 min and three times. Uranyl acetate (4% concentration) (Appendix A 5.2) was used for enbloc staining and incubating at room temperature in the dark for 1 h. Uranyl acetate was then removed. The cell pellet was washed twice with 1 mL of sterile distilled water for 10 min for each wash. Acetone series (v/v) (Appendix A 5.3) for dehydration were 20, 40, 60, 80, and 100%. The pellet was placed the first time in 20% acetone for 10 min and subsequently in 40, 60, and 80% acetone for 10 min, and twice in 100% acetone for 10 min. The cells were

spun down after each acetone treatment to remove the supernatant. Cells suspension were put on cover slips and allowed to completely dry, then put on stubs for PHA granule and cell morphology determination using SEM (JEOL JSEM-6460 LV, JEOL, Japan). Another set of dehydrated specimens was used for TEM preparation technique. The samples were infiltrated with 100% acetone and Epon (Appendix A 5.4). Ratios of acetone and Epon in infiltration were 2:1, 1:1, and 1:2. The ratios 2:1, and 1:1 were incubated at room temperature for 1 h, and then 1:2 was added and incubated for overnight at the same temperature. Each step of infiltration was removed the supernatant before changing to the next ratio. Then, low viscosity solutions were penetrated with pure Epon for 3 h. The solutions were transferred to beam capsules containing 100% low-viscosity embedding resin. The beam capsules were placed at 60°C for 24 h to allow to embedding. Ultrathin sections of the bacterial cells were prepared using ultra cut microtome (Ultracut RMC Boeckeler®, Boeckeler Instruments Inc., U.S.A.) with a Diatome diamond knife. The sections were picked up with 200-mesh copper grids coated with Formvar (0.3% [w/v] dissolved in ethylene dichloride) (Appendix A 5.5) and a layer of carbon. Grids with specimens were put on 5% uranyl acetate droplet on a piece of parafilm extended inside closed glass petri dish in the dark for 15 min, Then washed in sterile distilled water by dipping for several times, and put on tiny drop of 0.4% lead citrate (Appendix A 5.6) which surrounding with sodium hydroxide (NaOH) pellets on a piece of parafilm extended inside closed glass petri dish for 15 min. Grids were washed again in new clean water and placed in the grid box when completely dry. The sections were examined using TEM (JEOL JEM-1230, JEOL, Japan), and recorded of TEM images.

3.5 Monitoring of PHA accumulation in bacterial cells

PHA accumulation was monitored using Nile red dying technique during cultivation as described by Berlanga *et al*. (2006). Bacteria were grown overnight in TSB (Appendix A 1.8) at 30°C. Then, cultures were transferred into 100 mL flasks containing modified minimal medium (Appendix A 1.2) containing 0.5 μg/mL with adding of Nile red dye dissolved in dimethylsulfoxide. Liquid cultures were incubated in an orbital shaker (Heto Maxi-Shark, Heto-Holten, Gydevang, Denmark) at 100 rpm, 30°C for 4 days. At 0, 24, 48, 72, and 96 h, a 1-mL sample was removed, then centrifuged in a micro centrifuge at 10,000 rpm at room temperature for 5 min. Pellets were washed in 1 mL of PBS (pH 7.0), then suspended in 1 mL of 0.1 M glycine-HCl (pH 3.0), and incubated at room temperature in the dark for at least 2 h. The relative amount of PHA accumulated within bacterial cells, as indicated by the intensity of Nile-red orange fluorescence, was measured using spectrofluorophotometer (SHIMADZU RF-1501, SHIMADZU, Japan). The fluorescence excitation and emission wavelengths of the stained cells in 0.1 M glycine-HCl (pH 3) were 543 nm and 598 nm, respectively. Slits of excitation and emission were set to 10 nm at 900 V.

3.6 Identification of PHAs-producing bacteria

3.6.1 Morphological characterization

Bacterial isolates found to accumulate PHAs in their cells were identified by morphological and physiological characterization, and 16S rRNA gene sequence analysis as follow: morphological characterization including Gram stain reaction and cell morphology of bacterial isolates of PHAs-producing bacteria were determined and compared to data of known organisms described in the Bergey's

Manual of Systematic Bacteriology (Krieg *et al*., 2001), Microbiology: A Laboratory Manual (Cappuccino and Sherman, 1999), and Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

3.6.2 Physiological characterization

Some physiological characteristics were determined according to Krieg *et al*., (2001), Cappuccino and Sherman (1999), and Holt *et al*. (1994) as follows:

3.6.2.1 Motility test

Pure cultures of PHAs-producing bacteria were streaked on TSA (Appendix A 1.6) and incubated at 30°C for 16-18 h. Colonies were stabbed into motility medium (Appendix A 1.16) and incubated at 30°C for 24-48 h. The growth of bacteria out of needle stabbing indicated a positive test while no spreading growth indicates a negative test.

3.6.2.2 Nitrate reduction test

Pure cultures of PHAs-producing bacteria were streaked on TSA and incubated at 35°C for 16-18 h. The colonies were inoculated to the nitrate reduction broth (Appendix A 1.13) and incubated at 35°C for 48 h. Then, 1 mL of solution A (Appendix A 2.6) was added to each culture tube, followed by 1 mL of solution B (Appendix A 2.6) and mixed thoroughly. The development of a red color indicated a positive test while no red color developed indicates a negative test. The result of a negative test was confirmed by adding small amounts of zinc powder. The true negative test was indicated by the development of the red color.

3.6.2.3 Methyl red test

The overnight cultures of PHAs-producing bacteria on TSA were inoculated to MR-VP medium (Appendix A 1.11) and incubated at 35°C for 48 h. Five drops of the methyl red solution (Appendix A 2.5) were added directly to the broth. The development of a stable red color in the medium indicated a positive result.

3.6.2.4 Voges-Proskauer test

The overnight cultures of PHAs-producing bacteria on TSA were inoculated to MR-VP medium (Appendix A 1.11) and incubated at 35°C for 48 h. Five milliliters of solution A (Appendix A 2.7) were added to the medium, followed by 3 mL of solution B (Appendix A 2.7), then mixed gently to allow the medium to expose atmospheric oxygen, and left to be undisturbed for 30 min. A positive result was indicated by the development of a red color within 15 min or more but was not more than 1 h after reagents were added.

3.6.2.5 Indole formation

The overnight cultures of PHAs-producing bacteria on TSA were inoculated to LIM medium (Appendix A 1.10) and incubated at 35°C for 48 h. A few drops of Kovács' reagent solution (Appendix A 2.4) were then added. The positive result indicated by a bright fuchsia red color at the interface of the reagent and the semisolid medium within seconds after adding the reagent.

3.6.2.6 Carbohydrate fermentation

Acid production from carbohydrate tested using the method according to Chookietwattana (2003). Colonies of PHAs-producing bacteria grown on TSA at 35°C for 16-18 h were transferred to 5 mL of sterile phosphate buffer (Appendix A 2.10) to obtain a 4-6 McFarland unit suspension compared to McFarland turbidity standards (Appendix A 3.1). Then, 200 µL of McFarland unit suspension were transferred to 6 mL of phenol red broth (Appendix A 1.18) and mixed thoroughly. The 6.8% concentration of eight sugars (L-arabinose, D-fructose, D-galactose, D-glucose, lactose, D-mannitol, D-mannose, and D-xylose) were prepared by filtration through a 0.45 µm pore size membrane filter (Whatman, Puradisc^{TM}, England) onto the sterile container. Fifty microlitters of each sugar (for obtaining the final concentration of sugar at 2%) was distributed in each well of a sterile micro titer plate (recorded of which well contains which sugar). Then, 120 µL of the phenol red broth containing bacterial suspension was added to each carbohydrate in the micro-titer plate. The plate was incubated at 35°C for 24-48 h. Acid production was indicated by the color of the broth changing from red to yellow.

3.6.2.7 Starch hydrolysis

The overnight cultures of PHAs-producing bacterium were grown on starch agar (Appendix A 1.19) and incubated at 35°C for 48 h. The plate was then flooded with iodine solution (Appendix A 2.2.3). Blue color of the agar around the colony of the test organism indicated negative result (no starch hydrolysis), while a clear zone around the colony of the test organism indicated positive result (positive starch hydrolysis).

3.6.2.8 Gelatin hydrolysis

The overnight cultures of PHAs-producing bacteria was stabbed into gelatin medium (Appendix A 1.17) and incubated at 35°C for 48 h. Gelatin hydrolysis was indicated by the liquification of the medium after the tube was kept at 4°C for 30 min.

3.6.2.9 Lipid hydrolysis

The overnight cultures of PHAs-producing bacteria was grown on tributyrin agar (Appendix A 1.20) and incubated at 35°C for 48 h. Clear zone around the colonies was indicated that the secretion of lipase enzyme from bacteria (positive test).

3.6.2.10 Casein hydrolysis

The overnight cultures of PHAs-producing bacteria was grown on skimmed milk agar (Appendix A 1.21) and incubated at 35°C for 48 h. Clear zone around the colonies indicated the proteases activity from bacteria (positive test).

3.6.2.11 Hydrogen sulfide production

The overnight cultures of PHAs-producing bacteria was stabbed deep into the TSI agar (Appendix A 1.9) slant and also streaked on the surface of the slant, then incubated at 35°C for 48 h. Black color formed on the surface and along the stabbed line indicated the positive result.

3.6.2.12 Catalase test

The overnight cultures of PHAs-producing bacterial cells were transferred to the surface of a glass slide. One or 2 drops of 3% hydrogen peroxide (Appendix A 2.3) was added over the cells. Rapid appearance of gas bubbles indicated a positive result.

3.6.2.13 Oxidase test

The overnight cultures of PHAs-producing bacteria were streaked onto filter paper (Whatman no. 40) which was placed into a petri dish and wet with 0.5 mL of 1% dimethyl-p-phenylenediamine dihydrochloride (Appendix A 2.1). The development of a deep blue color at the inoculation site within 10 sec indicated a positive result.

3.6.2.14 Biochemical characterization using API identification system

Biochemical characteristics of PHAs-producing bacteria were also determined using API identification system (bioMérieur® Inc., France). API 50 CHB was selected for the identification of Gram-positive, rod and endospore-forming bacteria. API Listeria was used for the identification of Gram-positive, rod and non-sporing. Gram-positive cocci were determined by API 50 Staph. And API 20 NE was used for Gram-negative rod bacteria according to the manufacturer follows as:

(A) API 50 CHB

API 50 CHB was intended for the identification of *Bacillus* and related genera. It was a ready-to-use set of media which allow the fermentation of 49 carbohydrates. Pure cultures of PHAs-producing bacteria were streaked on TSA and incubated at 35°C for 16-18 h. A suspension with a turbidity equivalent to 2 McFarland (Appendix A 3.1) was prepared in the recommended medium. API 50 CHB strip was opened with an aseptic technique. Sterile distilled water was added to the tray of test kit. Then, the strips were cut and put on the tray with water. The bacterial suspension was filled in the API 50 CHB strip tubes. The kit was covered with lid and incubated at 35°C for 24-48 h. Results were observed at 24 h (± 2 h) and 48 (± 6 h) incubation. A positive test corresponds to acidification revealed by the phenol red indicator contained in the medium changing to yellow. For the esculin test (tube no. 25), a changed in color from red to black was observed. The results were recorded on recording sheet. The biochemical profile obtained for the strain after the final reading could identify using the identification software with bioMérieux, Inc. online database.

(B) API Listeria

API Listeria was invented for the identification of genus *Listeria*. The kit was composed of 10 tubes of dehydrated substrate. The overnight cultures of PHAs-producing bacteria on TSA (Appendix A 1.6) was used to prepared suspension with turbidity equal to 1 McFarland (Appendix A 3.1). API Listeria strip was opened with an aseptic technique. Three milliliters of sterile distilled water was added to the tray of test kit. Then, the strips were put on the tray with water. The

inoculum was filled into ESC to TAG strip tubes. For DIM tube, approximately of 100 µL of suspension was filled into the tube with a convex meniscus. The kit was covered with lid and incubated at 36°C (± 2°C) for 18-24 h. After the incubation period, a yellow or yellow-orange color of the results was indicated a positive reaction. Black color of ESC tube was positive test. For DIM tube, one drop of ZYM B was filled into the tube. After 3 min, an orange color was indicated a positive reaction. All of results were recorded on API Listeria recording sheet. The biochemical profile obtained for the strain after the final reading could identify by comparison to known *Listeria* sp. for API database (bioMérieux, Inc.).

(C) API Staph

API Staph was an identification system for the genera *Staphylococcus*, *Kocuria*, and *Micrococcus*. The overnight cultures of PHAs-producing bacteria on TSA (Appendix A 1.6) were used to prepared suspension with turbidity equal to 0.5 McFarland (Appendix A 3.1). API Staph strip was opened with an aseptic technique. Sterile distilled water was added to the tray of test kit. Then, the strips were put on the tray with water. The inoculum was filled in the strip tubes. Sterile emulsion oil (Appendix A 3.2) was dropped on ADH and URE test with a convex meniscus. The kit was covered with lid and incubated at 35°C for 24 h. Then, VP, NIT, and PAL tubes were added the specific reagents. One drop of VP1 and VP2 reagents were added to VP tube. After 10 min, a violet-pink color was indicated a positive reaction. A pale pink or light pink color obtained after 10 min was considered negative. NIT1 and NIT2 reagents were dropped on the NIT tube which was tested for nitrate reduction. A red color was indicated a positive reaction within 10 min. ZYME

A and ZYME B reagents were used for PAL test. A violet color was indicated positive reactions in 10 min. Results of carbohydrate utilization test were also observed after incubation. A red color of various sugar were indicated as positive, on the other hand yellow were negative. Orange-red of ADH test was found of arginine dihydrolase, and red-violet was indicated as urease. All of results were recorded on API staph recording sheet. The biochemical profile obtained for the strain after the final reading could identify by comparison to known organisms for API database (bioMérieux, Inc.).

(D) API 20 NE

API 20 NE is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods bacteria. The overnight cultures of PHAs-producing bacteria on TSA were used to prepared suspension with turbidity equal to 0.5 McFarland (Appendix A 3.1). API 20 NE strip was opened with an aseptic technique. Sterile distilled water was added to the tray of test kit. Then, the strips were put on the tray with water. The inoculum was filled in the strip tubes. Sterile emulsion oil (Appendix A 3.2) was dropped on the cupules of the 3 underlined tests, GLU, ADH, and URE until a convex meniscus is formed. The kit was covered with lid and incubated at 29°C (± 2°C) for 24 h (± 2 h). After the incubation period, one drop of NIT 1 and NIT 2 reagents were added into the NO_3 capsule for nitrate reduction test. After 5 min, a red color indicates a positive reaction to be recorded on the result sheet. One drop of JAMES reagent was added into TRP tube for indole formation test. The reaction takes place immediately, a pink color which develops in the whole cupule indicates a positive reaction. A yellow color and opaque of various

sugars were indicated as positive. All of results were recorded compared to known organisms for API database (bioMérieux, Inc.).

3.6.3 16S rRNA gene characterization

Sequencing of 16S rDNA was used as a tool for species identification. There are 5 major steps for 16S rDNA gene characterization of PHAs-producing isolates in this study: genomic DNA extraction, 16S rDNA amplification, PCR product purification, 16S rDNA cloning, 16S rDNA sequencing, and 16S rDNA sequence analysis and phylogenetic tree construction.

3.6.3.1 Genomic DNA extraction

Genomic DNA extraction from PHAs-producing isolates was performed as described by Cheng and Jiang (2006). The extraction method was the improved method of standard phenol-chloroform extraction. Selected bacteria were cultured in Lubia-Bertani (LB) broth (Appendix A 1.22) in rotary shaker at 250 rpm and 35°C for 16-18 h. One milliliter of cell suspension was centrifuged at 8,000 rpm for 2 min was used to pellet cells. After removing the supernatant, the cells were washed with 400 µL STE buffer (Appendix A 4.1) twice, and resuspended in 200 µL TE buffer (Appendix A 4.2). Then 100 µL Tris-saturated phenol (pH 8.0) (Appendix A 4.3) was added to the tubes, followed by a vortex-mixing step of 60 sec to lyse cells. The samples were subsequently centrifuged at 12,000 rpm for 5 min at 4°C to separate aqueous phase from organic phase. One hundred and sixty microliters of the upper aqueous phase were transferred to a clean 1.5 mL tube. TE buffer (40 µL) was added to make 200 µL and mixed with 100 µL of chloroform, then centrifuged for 5

min at 12,000 rpm at 4°C. Lysate was purified by chloroform extraction until a white interface was no longer present; this procedure might have to be repeated two to three times. The upper aqueous phase (160 µL) was transferred to a clean 1.5 mL tube. TE (40 µL) and 5 µL of RNase (10 mg/mL) were added and incubated at 37°C for 10 min to digest RNA. Then, 100 µL of chloroform was added to the tube, mixed well and centrifuged for 5 min at 12,000 rpm at 4°C. The upper aqueous phase (150 µL) was transferred to a clean 1.5 mL tube. The aqueous phase contained purified DNA was directly used for the subsequent experiments, or stored at -20°C. The purity and yield of the DNA were assessed spectrophotometrically by calculating the A260:A280 ratio and the A260 value to determine protein impurities and DNA concentrations.

3.6.3.2 16S rDNA amplification

The 16S rDNA of PHAs-producing bacteria was amplified by polymerase chain reaction (PCR) using primers fD1 and rP2 (Table 3.1). PCR product from 16S rRNA gene was about 1,500 bp. The PCR was performed using a thermocycler (ThermoHybaid PX2, Thermo Scientific, U.S.A.). An initial denaturation at 94°C for 2 min was followed by 35 cycles with denaturation at of 94°C (45 sec), annealing at 55°C (45 sec) and extension at 72°C (2 min), and a final extension at 72°C for 10 min. Each 25 µL reaction mixture contained 2.5 µL of genomic DNA (approximately 100 ng/µL), 14.25 µL of MilliQ water, 2.5 µL of 10× buffer (100 mM Tris-HCl, pH 9.1; 500 mM KCl and 0.1% Triton^{TM} X-100), 2 µL of $MgCl_2$ (25 mM), 0.25 µL of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), 1.25 µL of each primer (10 µM/µL), and 1 µL of *Taq* DNA polymerase (2 units/µL; Vivantis, VIVANTIS TECHNOLOGIES SDN BHD,

Malaysia). The PCR amplified products were examined by gel electrophoresis. Five microliters of PCR-amplified product was thoroughly mixed with 6X loading buffer (Appendix A 4.4). The mixture was loaded into the gel, and electrophoresis was carried out at constant 100 volts for 5 min and 60 volts for 40 min. DNA ladder (1 kbp; Fermentas International Inc., Canada) was used size reference of the PCR products. After electrophoresis, the agarose gel was stained with ethidium bromide (Appendix A 4.5) by soaking the gel in a solution containing 10 μg/mL of ethidium bromide, and visualized under UV transilluminator (BioRad UV-Transilluminator Universal Hood, BioRad, U.K.). The agarose gel was photographed for being reference.

Table 3.1 Oligonucleotide primers used for PCR amplification of 16S rDNA.

Primer	Primer sequence (5' to 3')	Target region[a]	Reference
fD1	AGAGTTTGATCCTGGCTCAG	339-357	Weisburg *et al.* (1991)
rP2	ACGGCTACCTTGTTACGACTT	1392-1406	Weisburg *et al.* (1991)

[a] *Escherichia coli* numbering

3.6.3.3 PCR product purification

The PCR products were purified prior to clone and sequence by using PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacture's protocol. The PCR clean up protocol was as follows: sample preparation, DNA binding, washing, and DNA elution. For sample preparation, 100 ml of PCR product were transferred to 1.5 mL microcentrifuge tube, and added with 500 μL of DF buffer. Then, the tube was mixed by vortex. For DNA binding, DF column was placed in a 2 mL collection tube. The mixed sample was transferred to

the DF column and spinned at full speed centrifugation for 30 sec. Flow-through was discarded and placed the DF column back in the 2 mL collection tube. In the washing step, 600 µL of wash buffer (ethanol mixed) was added into the center of the DF column and let stand for 1 min. Then, the column was placed for centrifuging at full speed for 30 sec. Flow-through was discarded and placed the DF column back in the 2 mL collection tube. The column was dried by centrifugation for 3 min at full speed. Dried DF column was transferred to a new 1.5 mL microcentrifuge tube for elution of DNA by adding 50 µL of elution buffer into the center of the column matrix, and let stand for 2 min. Purified DNA was eluted by centrifugation for 2 min at full speed. The purified PCR products were then transferred to new microcentrifuge tubes and stored at -20°C for 16S rDNA cloning.

3.6.3.4 16S rDNA cloning

The purifed 16S rDNA products were ligated into the T&A Cloning vector (RBC Bioscience, Taiwan) as described by the manufacturer. Briefly, the reaction was composed of 1 µL buffer A and B, TA cloning vector 2 µL, purified PCR product 1 µL, water 4 µL, and T4 ligase 1 µL, and incubated at 22°C in water bath for 2 h. Transformation of recombinant DNA into competent cells of *E. coli* DH5α (RBC Bioscience, Taiwan) was performed according to the manufacturer as follows: five milliliters of ligated TA cloning vector with 16S rDNA was mixed with 30 µL of competent cells and then placed on ice for 20 min. The reaction was transferred to 42°C in water bath for 1 min and placed on ice for 20 min. Two hundred micro litters of Soc Medium (RBC Bioscience, Taiwan) were added to the eppendrof tube of the reaction and inversed for 5 min. The tube was incubated at

37°C for 30 min in rotary shaker with 200 rpm speed. The reaction was poured on to LB agar plate mixing with isopropyl-beta-thio galactopyranoside (ITPG) and X-gal. Plate was incubated at 37°C overnight. White colonies were picked and checked for plasmid vectors and inserts DNA. Selected clones were streaked on LB agar containing 10 µL/mL ampicilin, and incubated at 37°C overnight, then transferred to LB broth containing 10 µg/mL ampicilin and incubated at 37°C over night in rotary shaker with 200 rpm speed. GeneJET^{TM} Plasmid Miniprep kit (Fermentas International, Inc., Canada) was used for plasmid extraction as described by the manufacturer. The clone cultures in LB broth were centrifuged at 6,000 rpm for 10 min. Supernatant was discardded. One milliliter of PE buffer was added in to the tube and then centrifuged at 6,000 rpm for 6 min. The solution was mixed and transferred to 1.5 mL microcentrifuge tube. The tube was centrifuged at 14,000 rpm for 2 min. Cell pellets were resuspended in 250 µL of resuspension solution. Lysis solution 250 µL was added and inversed; the solution became clear and viscous. Neutralization solution (350 µL) was added and the tube was inversed 4-6 times, then centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to GeneJET^{TM} column and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded. Wash solution (500 µL) was added to column, and centrifuged at 12,000 rpm for 30-60 sec, and the supernatant was discarded (repeated twice time of this step). The supernatant was discarded again and the column was place into centrifuge, and spun at 12,000 rpm for 1 min. The column was allowed to dry by open cap for 1 min, and placed in 1.5 mL microcentrifuge tube. Elution buffer (50 µL) was added at the central of the column and incubation at room temperature for 2 min. Microcentrifuge tube placed with column was centrifuged at 14,000 rpm for 2 min. The column was taken off from a

microcentrifuge tube. The supernatant in a microcentrifuge tube was used for DNA sequencing.

3.6.3.5 16S rDNA sequencing

The cloned ribosomal DNA was sequenced using the BigDye® Terminator Ready Reaction kit version 3.1 (BigDye®, Applied Biosystems, Inc., U.S.A.) according to the manufacturer's protocol. The 10 μL cycle sequencing reaction mixture contained 80-200 ng DNA, 4 μL BigDye, and 5 pM primer. Primers used for sequencing 16S rDNA were provided in the kit. The thermal profile consisted of 25 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. The cycle sequencing was performed in the thermal cycle GeneAmp® 9700 (ABI, Applied Biosystems, Inc., U.S.A.). The PCR mixtures were spun down briefly before DNA precipitation. The sequencing DNA fragments were precipitated by adding 16 μL of deionized water and 64 μL of 95% ethanol. The tube was vortexed briefly, incubated at 4°C for 15 min, and then spun at 12,000 rpm for 20 min at 4°C. The DNA pellet was washed with 300 μL of 70% ethanol, centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant was discarded. The DNA pellet was dried at room temperature in the dark. The sequencing gel used for an ABI 377 automated DNA sequencer (Perkin Elmer) was prepared as described in the manufacturer's protocol. The 6% polyacrylamide gel was casted in slab gel glass plates. The DNA pellet was dissolved in 3 μL of loading buffer, and loaded onto the gel. Electrophoresis was carried out at constant 750 volts for 8 h. Fluorescent signals were detected with ABI collection software. Base calling was performed using sequencing analysis software, and nucleotide sequence determination was performed using sequence navigator software.

The resulting sequences were assembled and manually corrected by using Chromas 1.56 program (Technelysium Pty. Ltd). The sequences obtained were deposited to the GenBank (NCBI, U.S.A.).

3.6.3.6 16S rDNA sequence analysis and phylogenetic tree construction

Nucleotide sequence data obtained from DNA sequencing software of ABI377 Automated DNA (Perkin Elmer). Sequence was interpreted and converted to single letter code in text file format by the Chromas 1.56 program. The sequence was also corrected by manual inspection of the chromatogram, and compared to local alignment search of the GenBank database using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnological Information (NCBI). (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic tree was inferred using the neighbour-joining method with software MEGA version 4 (Kumar, Tamura, and Nei, 2004). The stability relationships were evaluated by a boot strap analysis of 1,000 replications.

3.7 PHA production in controlled fermenter

Two and half liters of modified complex medium was sterilized in an autoclave at 121°C for 20 min. Fermentation was carried out in 6.6 L of controlled fermenter (Biostat® *B* plus, Sartorius BBI Systems GmbH, Melsungen, Germany). To keep the medium in the fermenter homologeneus, agitation was performed at 150 rpm. The medium was inoculated with 2% inoculums size of a fresh culture of the isolate CCA2-11 and cultured for 48 h. Then, 2.5 L of sterile modified minimal medium was

added and cultured for 48 h. The cultivation temperature was kept constant at 30°C. The pH was kept constant at 7.0 during fermentation through automatic addition of 3N NaOH and 3N HCl. Temperature, pH, and agitation were computer-controlled and monitored on line using MFCS SCADA Software (Sartorius, Germany). During cultivation, bacterial growth and PHA accumulation were measured at 0, 24, 48, 72, and 96 h. Cell cultures were prepared for PHA extraction according to Vanlautem and Gilain (1982) and Noda (1998). Bacterial culture in minimal medium for PHA extraction was harvested after cultured in minimal medium for 48 h and centrifuged at 8,500 rpm for 20 min at 4°C. The supernatant was removed. Cell pellet was washed with sterile 0.85% NaCl, and dried by freeze drying. Cell pellet was weighed and washed with 200 mL of methanol, and incubated at 100°C for 30 min. 1, 2-Dichloroethane was added to the pellet. The ratio of the solution and pellet was 20 mL per gram. Then, the mixing solution was transferred to round bottle flask for incubating in shaker incubator (Sheldon Model 1245 PC, Manufacturing, Inc., U.S.A.) at 70-90°C for 30 min. The solution was filtrated through cotton wool, then kept the filtrated solution at 4°C until gel setting. The gel was separated by 1, 2-Dichloroethane using evaporator (A Büchi Rotavapor R-200, BÜCHI Labortechnik AG, Switzerland) at 80°C and 300 mbar pressure. The weight of PHAs was recorded. PHA precipitate was washed by chloroform and precipitated in methanol. The PHA solution was filtrated and incubated at 80°C for 1 h.

3.8 Maintenance of selected bacterial isolates for future application

PHAs-producing bacteria isolated from cassava pulp were re-streaked on TSA plates and incubation at 35°C for 16-18 h. After incubation, the bacterial strains were transferred to TSB and incubated at the same conditions. The isolates were preserved in 5% skim milk (final concentration) and kept at -80°C.

3.9 Data analysis

All results of physiological traits and 16S rDNA characterizations of PHAs-producing bacteria were analyzed and concluded. The physiological characteristics of all isolates in the study were compared to physiological data of known bacteria described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and API data base (bioMérieur, Inc., France). Bacterial strains were selected for 16S rDNA characterization. Genetic relationships were determined to the degree that statistical support for branches in the phylogeny, was provided. The diversity of PHAs-producing bacteria isolated from cassava pulp samples was concluded.

CHAPTER IV

RESULTS AND DISCUSSION

Seven hundred and seventy two bacteria isolated from cassava pulp were examined for the PHA accumulation within their cells. PHAs-producing bacteria were identified by their morphological and physiological characteristics. The ribosomal gene characterizations of some selected strains were described. The diversity of PHAs-producing bacteria isolated from cassava pulp samples was concluded. In addition, PHA production by a selected strain in controlled fermenter was investigated. Results achieved are presented and discussed in these following sections.

4.1 Selection of PHAs-producing bacteria isolated from cassava pulp

4.1.1 Collection of PHAs-producing bacteria isolated from cassava pulp

Sources of bacteria isolated from cassava pulp samples were the Microbial Culture Collection and Applications Research Unit, Institute of Science, Suranaree University of Technology, and new isolates from cassava pulp samples collected from modified cassava starch industrial factories, and sun drying fields for cassava pulp. Seven hundred and seventy two bacteria isolated from cassava samples were examined for the PHA accumulation within their cells (Table 4.1).

Table 4.1 Sources of bacteria isolated from cassava pulp samples.

No.	Sources of bacteria	Number of isolates
1	Microbial Culture Collection and Applications Research Unit, Institute of Science, Suranaree University of Technology	422
2	Cassava pulp samples collected from modified cassava starch industrial factories (3 factories)	182
3	Sun drying field for cassava pulp collected from 2 fields	168
	Total	722

4.1.2 Bacterial isolation

Bacteria were isolated from cassava pulp samples using five difference agar media CMC, TCMC, PCA, SA, and TSA (example of bacterial colonies as shown in Figure 4.1). Forty one isolate codes were selected for PHAs-producing bacteria according to their source of sampling and culturing media (Table 4.2). Seven hundred and seventy two bacterial isolates obtained from different isolation sources (Figure 4.2) were investigated for PHAs-accumulation in their cells.

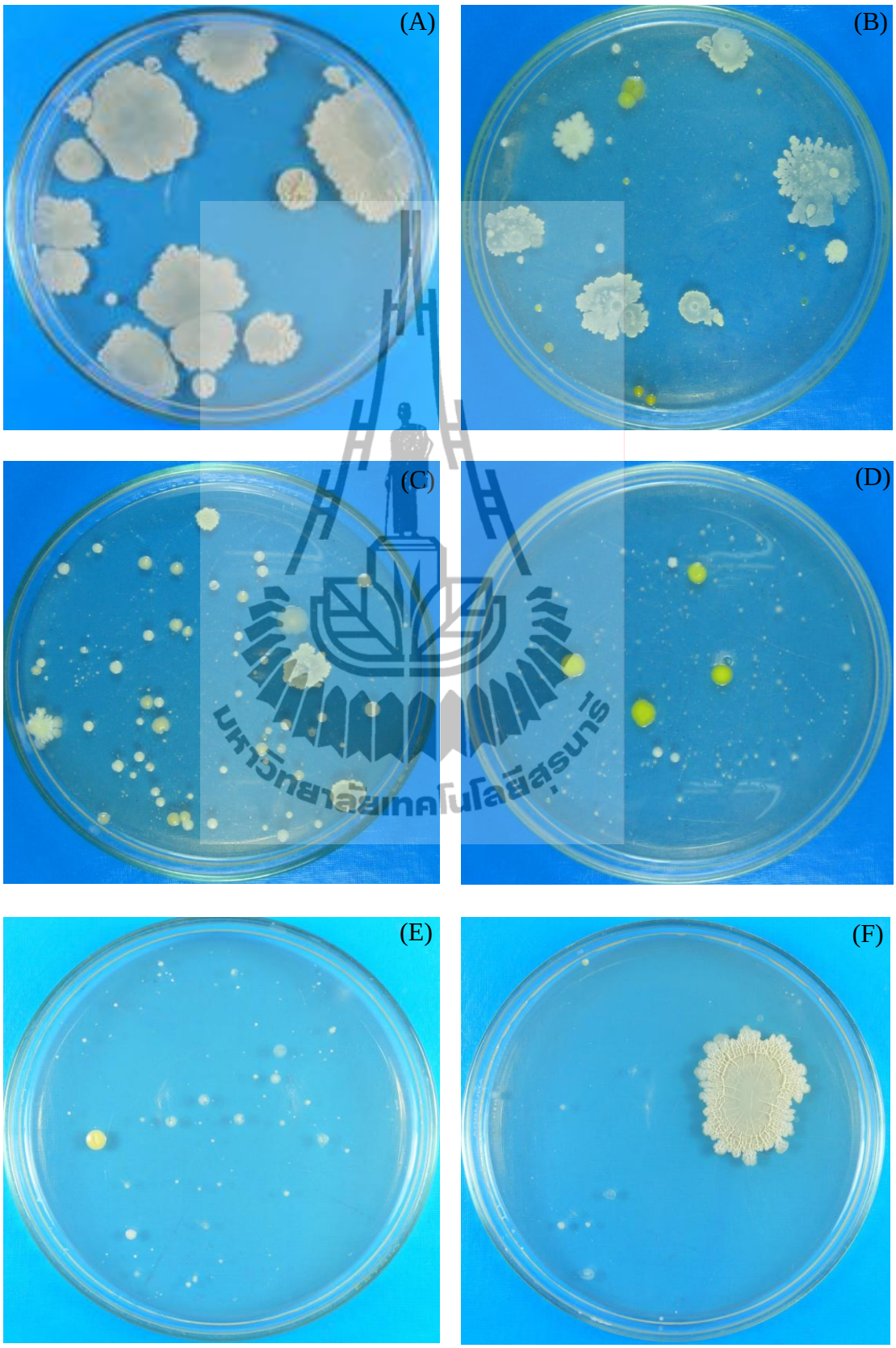


Figure 4.1 Examples of bacterial colonies isolated from cassava pulp samples on TSA (A), PCA (B), CMC (C), SA (D), and TCMC (E, F) media.

Table 4.2 Codes of selected bacteria isolated from cassava pulp.

No.	Isolate codes	Source of cassava pulp sample	Culture[a] medium	Number of bacteria
1	CAC	Modified cassava starch factory No. 1	CMC	36
2	CACT	Modified cassava starch factory No. 1	TCMC	30
3	CAP	Modified cassava starch factory No. 1	PCA	11
4	CAS	Modified cassava starch factory No. 1	PCA	30
5	CASA	Modified starch factory No. 1	SA	66
6	CAT	Modified cassava starch factory No. 1	TSA	12
7	CSC 1	Modified cassava starch factory No. 1 Plot 1	CMC	6
8	CSC 2	Modified cassava starch factory No. 1 Plot 2	CMC	6
9	CSCT 2	Modified cassava starch factory No. 1 Plot 2	TCMC	6
10	CSP 1	Modified cassava starch factory No. 1 Plot 1	PCA	28
11	CSP 2	Modified cassava starch factory No. 1 Plot 2	PCA	40
12	CSSA 1	Modified cassava starch factory No. 1 Plot 1	SA	3
13	CST 1	Modified cassava starch factory No. 1 Plot 1	TSA	16
14	CST 2	Modified cassava starch factory No. 1 Plot 2	TSA	26
15	CWC 1	Modified cassava starch factory No. 1 Plot 1	CMC	15
16	CWCT 1	Modified cassava starch factory No. 1 Plot 1	CMC	17
17	CWCT 2	Modified cassava starch factory No. 1 Plot 2	TCMC	5
18	CWP 1	Modified cassava starch factory No. 1 Plot 1	PCA	16
19	CWP 2	Modified cassava starch factory No. 1 Plot 2	PCA	26
20	CTK	Modified cassava starch factory No. 2	TSA	34
21	SC	Modified cassava starch factory No. 1	PCA	27
22	CCS1	Modified cassava starch factory No. 1	CMC	15
23	CCS2	Modified cassava starch factory No. 1 Plot 2	CMC	26
24	CCS3	Modified cassava starch factory No. 1 Plot 3	CMC	26
25	CCA1	Modified cassava starch factory No. 3	CMC	17
26	CCA2	Modified cassava starch factory No. 3	CMC	11
27	WCC1	Sun drying field for cassava pulp No. 1 Plot 1	CMC	11
28	WCC2	Sun drying field for cassava pulp No. 1 Plot 2	CMC	8
29	BCC1	Sun drying field for cassava pulp No. 2 Plot 1	CMC	10
30	BCC2	Sun drying field for cassava pulp No. 2 Plot 2	CMC	17
31	BCC3	Sun drying field for cassava pulp No. 2 Plot 3	CMC	20
32	CCP1	Modified cassava starch factory No. 1 Plot 2	PCA	15
33	CCP2	Modified cassava starch factory No. 1 Plot 2	PCA	19
34	CCP3	Modified cassava starch factory No. 1 Plot 3	PCA	9
35	PCA1	Modified cassava starch factory No. 2 Plot 1	PCA	13
36	PCA2	Modified cassava starch factory No. 2 Plot 2	PCA	17
37	WPC1	Sun drying field for cassava pulp No. 1 Plot 1	PCA	13
38	WPC2	Sun drying field for cassava pulp No. 1 Plot 2	PCA	17
39	BPC1	Sun drying field for cassava pulp No. 2 Plot 1	PCA	34
40	BPC2	Sun drying field for cassava pulp No. 2 Plot 2	PCA	8
41	BPC3	Sun drying field for cassava pulp No. 2 Plot 3	PCA	10
		Total of the bacterial isolates		722

[a] CMC: carboxymethylcellulose agar; TCMC: thermo-carboxymethylcellulose agar; PCA: plate count agar; SA: starch agar; and TSA: trypticase soy agar.

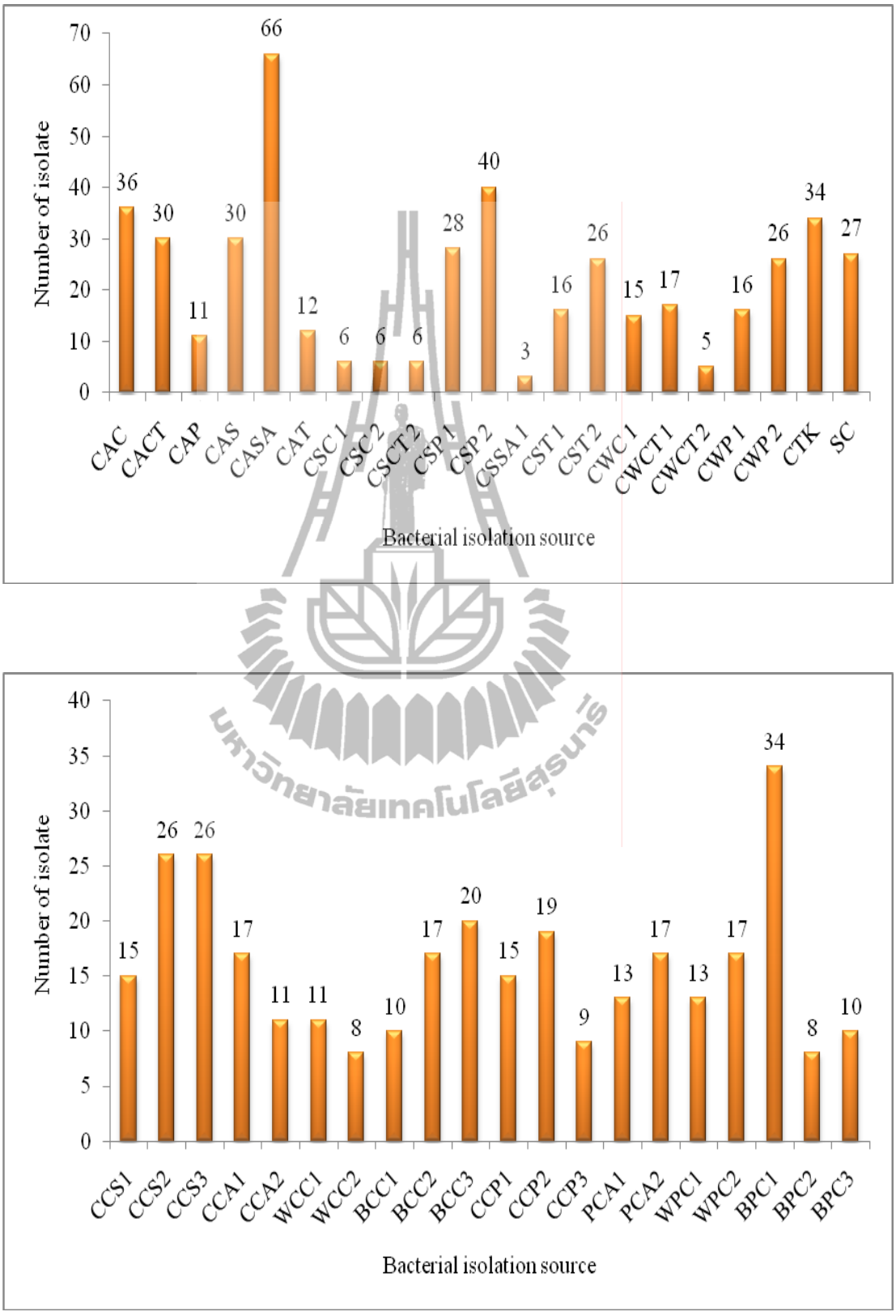


Figure 4.2 Number of bacterial isolates obtained from different isolation sources for investigating PHA accumulation in their cells.

Colony morphology (colony edge, surface, whole colony, elevation, and diameter of colony) of bacteria selected for PHAs accumulation investigation was determined (Table 4.3). Gram morphology (Gram staining, cell shape, cell size, and spore-forming) was also observed (Figure 4.3).

Table 4.3 Colony and cell morphology of bacteria isolated from cassava pulp samples used for detection of PHA accumulation in their cells.

Colony/cell morphology		Proportion of 772 isolates (%)
Colony morphology		
Whole colony	Circular	70
	Irregular	24
	Functiform	5
	Rhizoid	1
Colony edge	Entire	79
	Lobate	20
	Undunate	1
Colony surface	Smooth	70
	Rough	30
Colony elevation	Umbronate	37
	Rised	28
	Convex	23
	Flat	7
	Pulvinate	5
Colony diameter	Ranging from 0.1 to 1.2 cm	
Cell morphology		
Gram's reaction	Gram-positive	98
	Gram-negative	2
Cell shape	Rod	85
	Cocci	15
Endospore	Spore-forming	51
	Non spore-forming	49
Cell size		
Gram-positive rod non-sporing	0.3-0.5×2.0-2.4 to 0.5-0.8×2.2-3.1 µm	
Gram-negative rod	0.2-0.5×1.2-1.3 to 0.5-0.7×1.7-3.1 µm	
Gram-positive rod endospore-forming	0.4-0.7×1.1-1.4 to 0.77-1.05×3.67-6.38 µm	
Gram-positive coccus	0.18-0.28×0.18-0.28 to 0.4-1.2×0.4-1.2 µm	

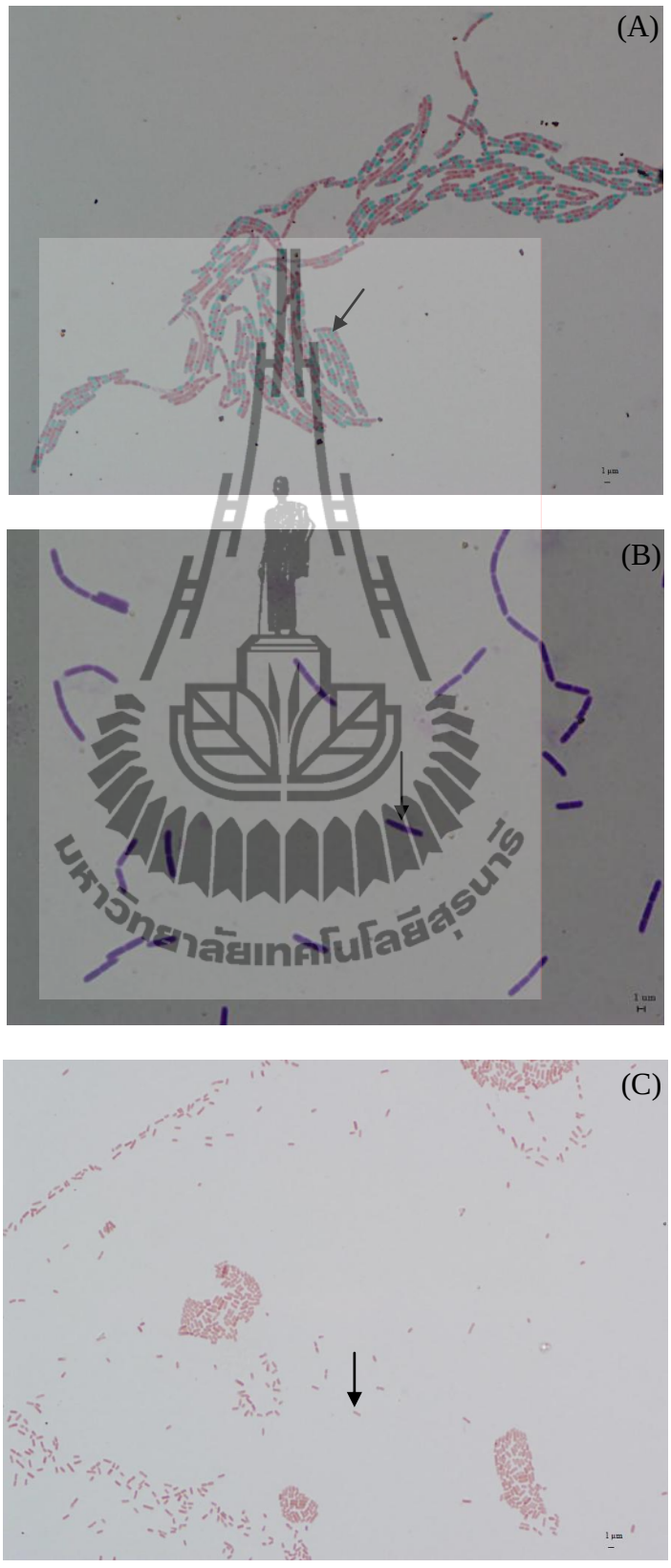


Figure 4.3 Representative of endopore-forming (arrows, A), Gram-positive (B) and Gram-negative (C) rod bacteria isolated from cassava pulp.

Seven hundred and seventy two isolates of bacteria isolated from cassava pulp were obtained from the Microbial Culture Collection and Application Research Unit. The majority of colonies were circular (70%), entire edge (79%), smooth surface (70%), umbronate elevation (37%), and 0.1-0.3 cm in diameter. For cell morphology, Gram-positive (98%), rods shape (85%) and endospore-forming (51%) bacteria were characterized. Sizes of these bacterial cells ranged from 0.18-0.28×0.18-0.28 to 0.77-1.05×3.67-6.38 µm. These isolates were re-streaked on the original agar medium for bacterial purification. Pure cultures were kept in 5% skim milk (final concentration) at -80°C and using for the detection of PHA accumulation in their cells. Bacteria isolated from cassava products such as Attiéké (a fermented cassava root or pulp) (Coulin *et al*., 2006), Fufu (a cassava creamy white fermented paste) (Fagbemi and Ijah, 2006), and cassava-cauim (beverage) (Almeida, Rachid, and Schwan, 2007) were Gram-positive rods belonging to genera *Bacillus*, *Leuconostoc*, *Lactobacillus*, and *Paenibacillus* that have been reported.

4.1.3 Development of cultivation media

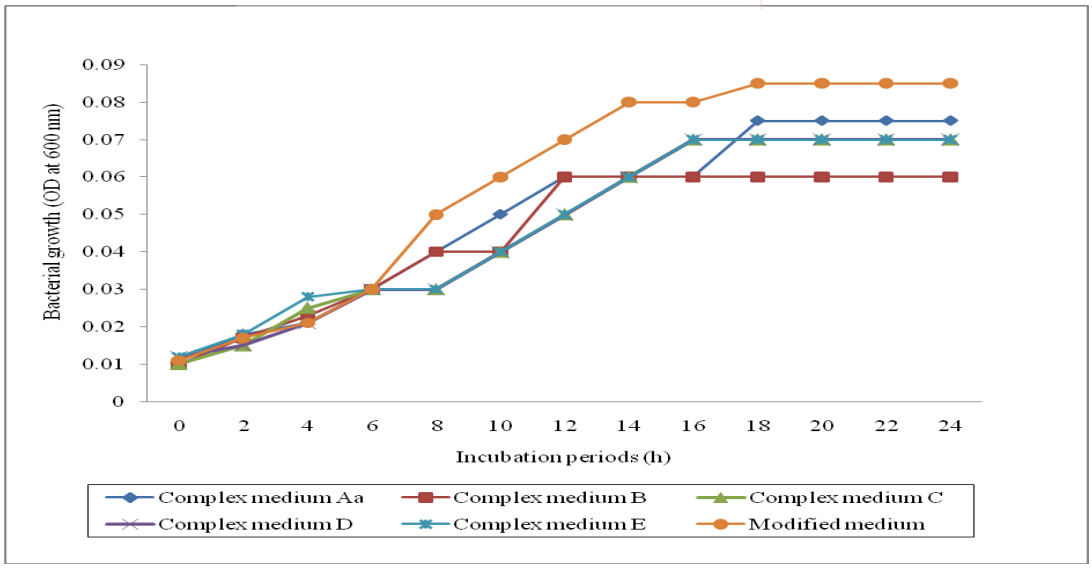
Development of culture media for PHAs-producing bacteria was studied to obtain the appropriate media for the detection of these bacteria. Major and minor elements in complex and minimal media were investigated using data from relevant references (Tables 4.4-4.6). Twenty bacterial isolates from cassava pulp were tested for their growth in the investigated media. It was found that the suitable complex medium was composed of (per liter) 5 g yeast extract, 5 g polypeptone, 5 g tryptone, 2.5 g NaCl, and 10 g glucose (Table 4.4). In this study, the medium could promote bacterial cell growth within 10 h of incubation. Comparison of bacterial cell growth

between complex medium from references and modified medium in this study was shown on Figure 4.4.

Table 4.4 Complex medium for the detection of PHAs-producing bacteria isolated from cassava pulp.

Component (g/L)	Complex medium from reference[a]					Medium modified for this study
	A	B	C	D	E	
Yeast extract	2	5	5	10	5	**5**
Meat extract	3			5		
Beef extract			2.5			
Polypeptone	5			10		**5**
Tryptone		10			8	**5**
Trypticase						
Peptone			5			
Phytane						
NaCl	2	5			2.5	**2.5**
$(NH_4)_2SO_4$			2.5	5		
Glucose		10				**10**

[a]A: Tajima *et al.* (2003); B: Lee *et al.* (1994); C: Yu *et al.* (2002); D: Fukui *et al.* (1998); and E: Kung *et al.* (2007).



[a]A: Tajima *et al.* (2003); B: Lee *et al.* (1994); C: Yu *et al.* (2002); D: Fukui *et al.* (1998); and E: Kung *et al.* (2007).

Figure 4.4 Optical density of bacterial growth in difference complex medium incubated at 30°C for 0-24 h.

The suitable minimal medium was found to compose of 0.01 g $CaCl_2 \cdot 2H_2O$, 0.05 g ferrous ammonium citrate, 10 g glucose, 1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 g Na_2HPO_4, 1 g $(NH_4)_2SO_4$, 1 mL of trace element solution (0.2 g $CoCl_2 \cdot 6H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 5.56 g $FeSO_4 \cdot 7H_2O$, 0.3 g H_3BO_3, 0.03 g $MnCl_2 \cdot 4H_2O$, 0.03 g $NaMoO_4 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$) (Table 4.5, and 4.6). Some major elements for culturing PHAs-producing bacteria isolated from cassava pulp were further optimized. Phosphate has been reported to be a crucial element stimulating PHA accumulation in bacterial cell (Shi, Lee, and Ma, 2007). Phosphate in the form of potassium phosphate (KH_2PO_4) and disodium phosphate (Na_2HPO_4) was varied in its concentrations (Table 4.7). The addition of 1 g/L of KH_2PO_4 and 3 g/L of Na_2HPO_4 in to optimized minimal medium was found to support the maximum accumulation of PHAs of bacteria isolated from cassava pulp. Gram-positive, rod, and endospore-forming bacteria were the majority group isolated from cassava pulp. Full, Jung, and Madigan (2006) reported the modified medium for detection of PHA in *Bacillus* cells.

Table 4.5 Optimized minimal medium for the detection of PHAs-producing bacteria isolated from cassava pulp.

Component	Concentration (g/L) from reference[a]												Optimized medium
	A	B	C	D	E	F	G	H	I	J	K	L	
Agar										15			**15**
$CaCl_2$										0.01			
$CaCl_2 \cdot 2H_2O$	0.01		0.075	0.1		0.7		0.01				0.01	**0.01**
EDTA						0.1							
Ferrous ammonium citrate							0.05			0.06		0.06	**0.05**
$FeSO_4 \cdot 7H_2O$	0.02												
Glucose	10									5			**10**
K_2HPO_4						0.92			5.8				
KH_2PO_4	0.83	2.65	1	2.3	1.4	0.45	1.4	2.3	3.7	1.5	2.65	1.5	**1**
$MgSO_4 \cdot 7H_2O$		0.4			0.3		0.3						
100 mM $MgSO_4$									0.24				
$MgSO_3 \cdot 7H_2O$	0.2		0.2					0.25					
$MgSO_4 \cdot 7H_2O$				0.25		0.6				0.2	0.4	0.2	**0.2**
$NaHCO_3$				0.3			0.5	0.3					
Na_2HPO_4		3.8			3		1.4						**3**
$Na_2HPO_4 \cdot 7H_2O$										6.7		6.7	
$Na_2HPO_4 \cdot 12H_2O$	3.32							7.3			9.65		
Na_2HCO_3					0.3								
Sodium acetate						2.4							
NH_4Cl						0.16				0.1			
$(NH_4)_2SO_4$	2		0.75		3		1.5	0.66			0.5	0.5	**1**
$(NH_4)H_2PO_4$									5.94				
Nile red										0.0005			**0.0005**
Yeast extract					1		0.3						
Na_2HCO_3					0.3								

[a]A: Lee *et al*. (1994); B: Yu *et al*. (2002); C: Full *et al*. (2006); D: Ciesielski *et al*. (2007); E: Kojima *et al*. (2004); F: Halet *et al*. (2007); G: Takagi *et al*. (2004); H: Kim *et al*. (2000); I: Foster *et al*. (2005); J: Berlanga *et al*. (2006); K: He *et al*. (1998); and L: Zheng *et al*. (2005).

Table 4.6 Optimized trace element solution for the detection of PHAs-producing bacteria isolated from cassava pulp.

Component	Trace elements (g/L) from reference[a]										
	A	B	C	D	E	F	G	H	I	J	**Optimized trace element**
$CaCl_2$				9							
$CaCl_2 \cdot 2H_2O$								1.67			
$CaCl_2 \cdot H_2O$									10		
$CoCl_2 \cdot 6H_2O$	0.2				0.15	2					**0.2**
$CoCl_2 \cdot H_2O$										0.2	
$CoSO_4 \cdot 7H_2O$			5.62				5.62	2.81			
$CuCl_2 \cdot 2H_2O$			0.34				0.34	0.117			
$CuSO_4 \cdot 5H_2O$	0.01	0.002			0.03	0.1			0.03	0.01	**0.01**
$Fe(NH_4)SO_4$		0.2									
$FeCl_3 \cdot 6H_2O$					1.5				20		
$FeSO_4 \cdot 7H_2O$			5.56	9			5.56	2.78			**5.56**
H_3BO_3	0.3		0.6		0.15	3	0.6			0.3	**0.3**
$MnCl_2 \cdot 2H_2O$				1.5							
$MnCl_2 \cdot 4H_2O$	0.03	0.005	3.96	0.15	0.12	0.3	3.96	1.98	0.05	0.03	**0.03**
$NaBO_4O_2 \cdot 10H_2O$		0.002									
$NaMoO_4 \cdot 2H_2O$	0.03	0.002	0.06		0.06	0.2	0.06			0.03	**0.03**
$NiCl_2 \cdot 6H_2O$	0.02		0.04				0.04			0.02	**0.02**
$NiSO_4 \cdot 7H_2O$						0.28					
$ZnSO_4$								0.29			
$ZnSO_4 \cdot 7H_2O$	0.1	0.005	0.58	1.5	0.12	1	0.58		0.1	0.1	**0.1**

[a]A: Lee *et al*. (1994); B: Yu *et al*. (2002); C: Ciesielski *et al*. (2007); D: Kojima *et al*. (2004); E: Halet *et al*. (2007); F: Takagi *et al*. (2004); G: Kim *et al*. (2000); H: Foster *et al*. (2005); I: He *et al*. (1998); and J: Zheng *et al*. (2005).

Table 4.7 Variation of phosphate concentration for minimal medium for the detection of PHAs-producing bacteria isolated from cassava pulp.

Component	Concentration (g/L)									
	A	**B**	C	D	E	F	G	H	I	J
Agar	15	**15**	15	15	15	15	15	15	15	15
$CaCl_2$ $2H_2O$	0.01	**0.01**	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Ferrous ammonium citrate	0.05	**0.05**	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Glucose	10	**10**	10	10	10	10	10	10	10	10
KH_2PO_4	1.4	**1**	0.83	0.45	0.1	1.5	1.5	1.5	1.5	0.83
$MgSO_4 \cdot 7H_2O$	0.2	**0.2**	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Na_2HPO_4	3	**3**	3	3	3	2.5	2	1.66	1.4	1.66
$(NH_4)_2SO_4$	1	**1**	1	1	1	1	1	1	1	1

Comparison of PHA accumulation in bacterial cells between minimal medium from references and modified medium in this study were shown on Table 4.8 and Figure 4.5.

Table 4.8 PHA accumulation in bacterial cells compared between minimal medium from references, negative control (*E. coli* TISTR 527), and optimized medium in this study.

Minimal medium	References	PHA accumulation (%)
		Bacterial isolates when stained with 1% Nile blue A, observed under fluorescent microscope, and measured by Immage-ProPlus® 6.0 Program
A	Lee *et al*. (1994)	51
B	He *et al*. (1998)	62
C	Kim *et al*. (2000)	70
D	Yu *et al*. (2002)	80
E	Kojima *et al*. (2004)	17
F	Takagi *et al*. (2004)	69
G	Foster *et al*. (2005)	53
H	Zheng *et al*. (2005)	15
I	Full *et al*. (2006)	82
J	Berlanga *et al*. (2006)	68
K	Halet *et al*. (2007)	75
L	Ciesielski *et al*. (2007)	53
M	*E. coli* TISTR 527	-
N	Optimized medium	85

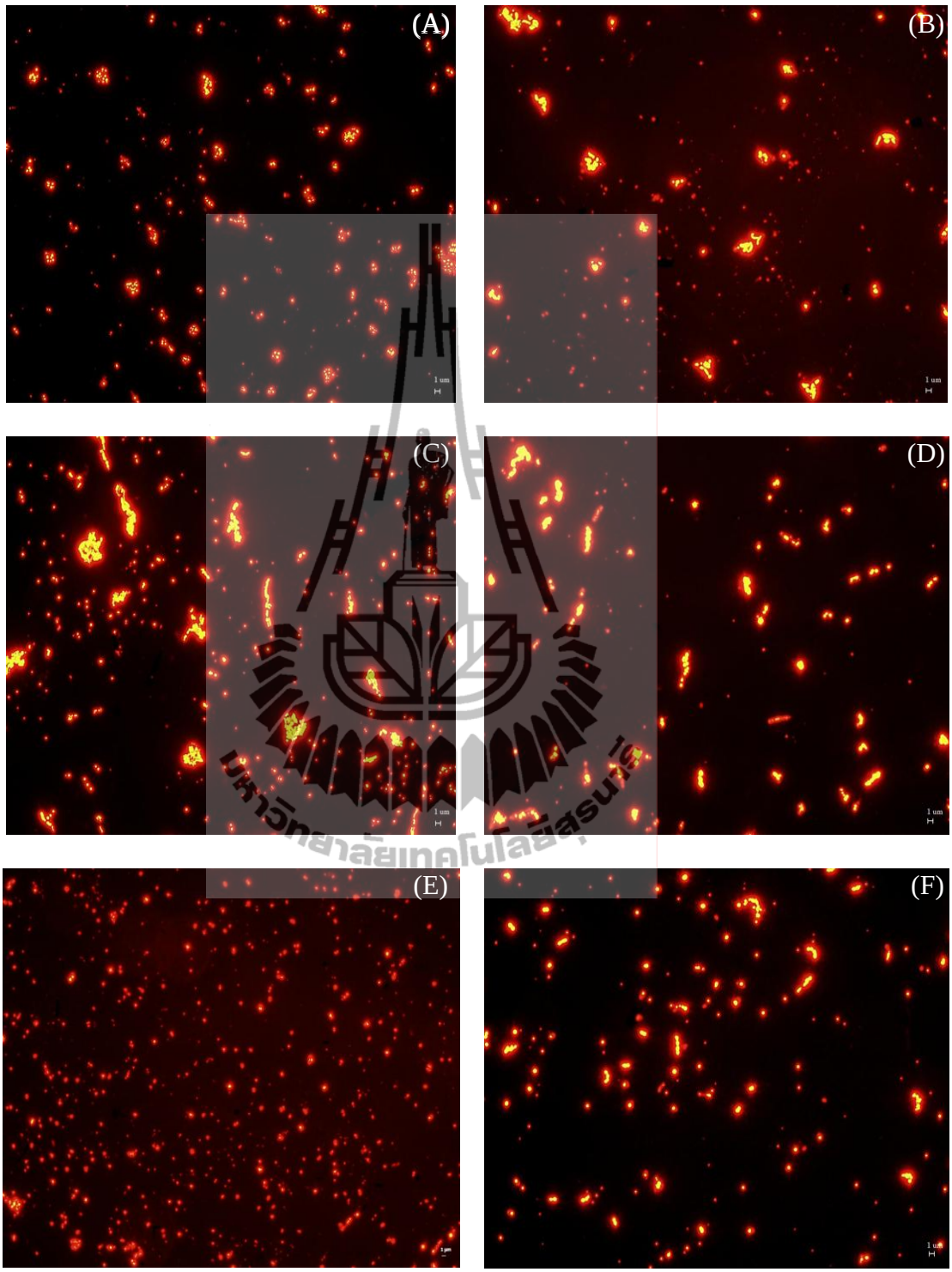


Figure 4.5 PHA accumulation in bacterial cells compared between minimal medium from references (Lee *et al*. (1994) (A); He *et al*. (1998) (B); Kim *et al*. (2000) (C); Yu *et al*. (2002) (D); Kojima *et al*. (2004) (E); Takagi *et al*. (2004) (F); Foster *et al*. (2005) (G); Zheng *et al*. (2005) (H); Full *et al*. (2006) (I); Berlanga *et al*. (2006) (J); Halet *et al*. (2007) (K); and Ciesielski *et al*. (2007) (L)), negative control (*E. coli* TISTR 527) (M), and modified medium in this study (N).

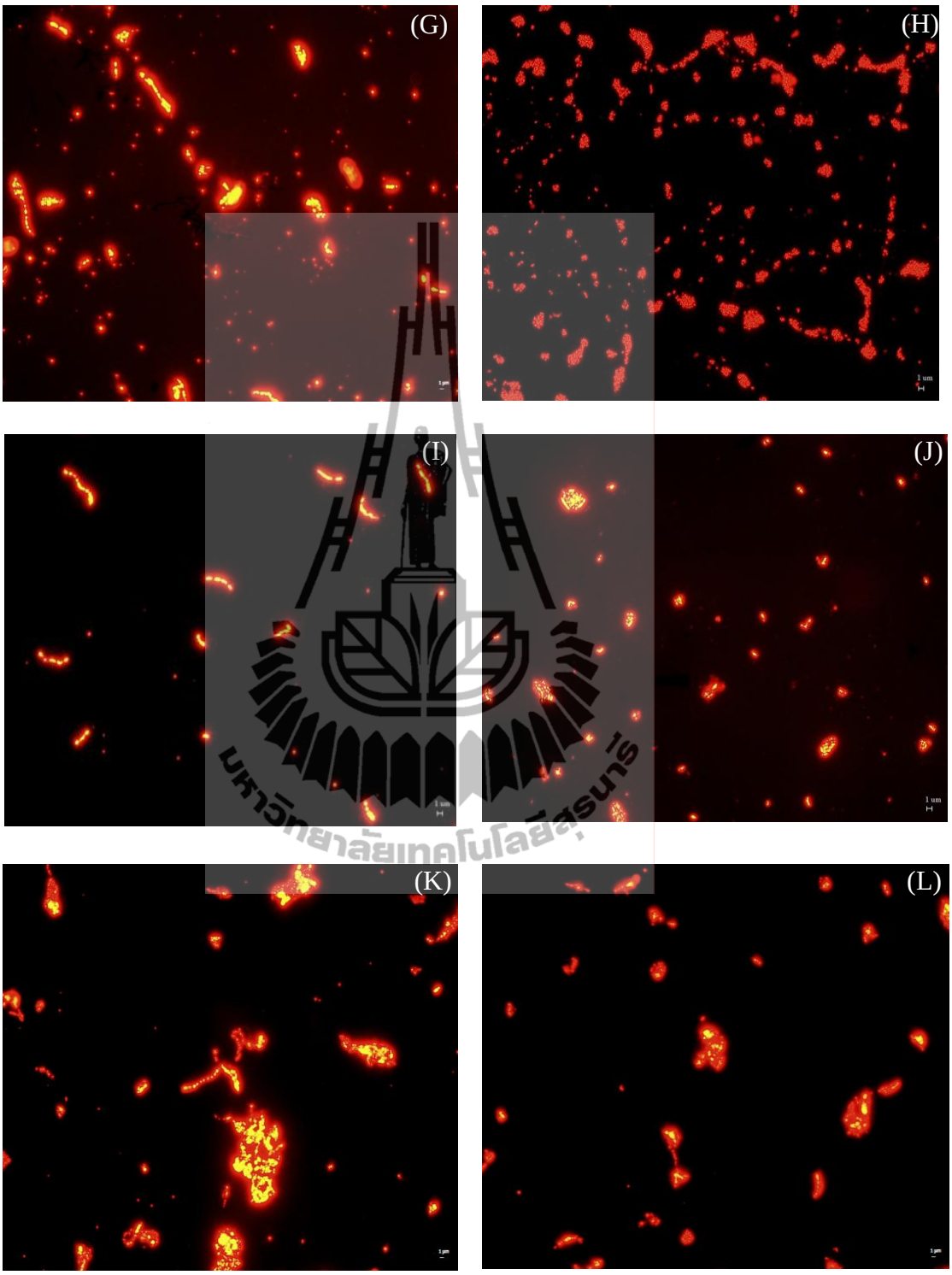


Figure 4.5 (Continued) PHA accumulation in bacterial cells compared between minimal medium from references (Lee *et al*. (1994) (A); He *et al*. (1998) (B); Kim *et al*. (2000) (C); Yu *et al*. (2002) (D); Kojima *et al*. (2004) (E); Takagi *et al*. (2004) (F); Foster *et al*. (2005) (G); Zheng *et al*. (2005) (H); Full *et al*. (2006) (I); Berlanga *et al*. (2006) (J); Halet *et al*. (2007) (K); and Ciesielski *et al*. (2007) (L)), negative control (*E. coli* TISTR 527) (M), and modified medium in this study (N).

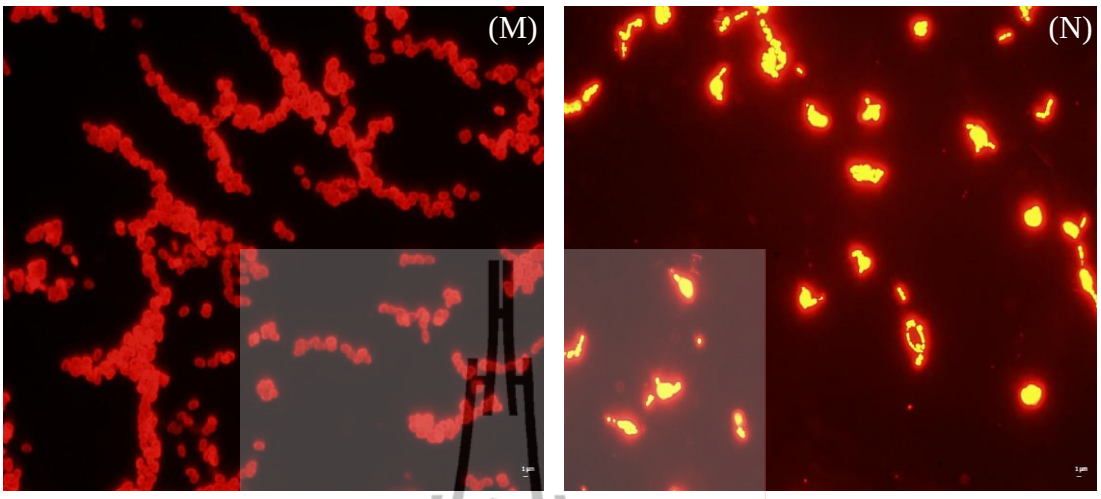


Figure 4.5 (Continued) PHA accumulation in bacterial cells compared between minimal medium from references (Lee *et al*. (1994) (A); He *et al*. (1998) (B); Kim *et al*. (2000) (C); Yu *et al*. (2002) (D); Kojima *et al*. (2004) (E); Takagi *et al*. (2004) (F); Foster *et al*. (2005) (G); Zheng *et al*. (2005) (H); Full *et al*. (2006) (I); Berlanga *et al*. (2006) (J); Halet *et al*. (2007) (K); and Ciesielski *et al*. (2007) (L)), negative control (*E. coli* TISTR 527) (M), and modified medium in this study (N).

4.1.4 Screening of PHAs-producing bacteria

Nile red dyeing technique was used for screening of PHAs-producing bacteria isolated from cassava pulp. The purified isolates were cultured on complex agar medium, then transferred to minimal agar medium which was supplemented with 0.5 mg/L Nile red (dissolved in 1 mL of dimethylsulfoxide) (Berlanga *et al*., 2006), then incubated at 30°C for 24 to 48 h. Development of pink colonies under UV light after cultivation on minimal medium were selected for microscopic detection of PHA. *Alcaligenes eutrophus* TISTR 1095 was used as the positive control of PHAs-producing strain. *Escherichia coli* TISTR 527 was utilized for negative control (Figure 4.6). In this study, Nile red dyeing technique was efficient for screening PHAs-producing bacteria isolated from cassava pulp samples. The method was low cost, less labor and time consuming. It was the preliminary method for rapid detection of PHAs-producing bacteria isolated from cassava pulp, and the result could be read

within 24-48 h under UV light whereas some other methods for screening of PHAs-producing bacteria. Such as the method used for screening bacteria isolated from microbial mats Ebro Delta, Spain (Berlanga *et al*., 2006), could detected positive colonies up to 5 days of incubation periods. Total of 772 isolates isolated from cassava pulp samples in this research, the technique could screen PHAs-producing isolates within a week. Eighty two isolates could develop of pink colonies under UV light at 280 nm wave length, which were selected for the detection of PHAs-producing bacteria and PHA accumulation in their cells.

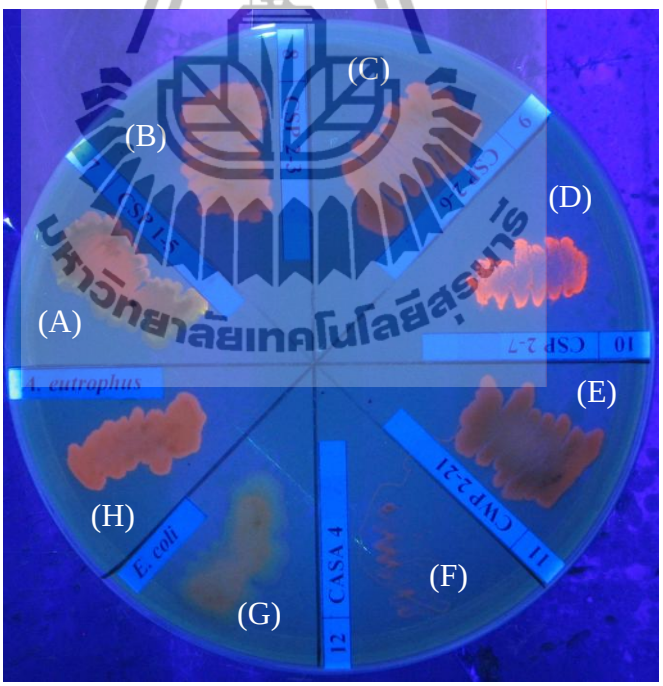


Figure 4.6 Representative of pink colonies of PHAs-producing bacteria: CSP1-5(A) CSP2-3 (B), CSP2-6 (C), CSP2-7 (D), CWP2-21 (E), CASA4 (F), *E. coli* TISTR 527 (G), and *A. eutrophus* TISTR 1095 (H) cultured on modified minimal medium supplemented with 0.5 mg/L Nile red and incubating at 30°C for 48 h.

4.2 Detection of PHAs-producing bacteria

4.2.1 Fluorescent microscope for the detection of PHAs-producing bacteria

PHAs-producing bacteria isolated from cassava pulp were detected by Nile blue A dyeing technique and observed under fluorescence microscope with an excitation wavelength of approximately 650 nm. Stains produced PHA in their cell were given bright orange (Figure 4.7). Percentages of PHA accumulation were calculated from bacterial cell area stained with Nile blue A. The cell and PHA areas were measured by Immage-Pro Plus Version 6.0.0.Z60 (Media Cybematics, Inc., Japan). PHA accumulation in bacterial cells was depended on capability of each strain and appropriated medium which were developed (as previous described in section 4.1.3) (Tables 4.4-4.7). Strains CST2-45-1-1, CST2-2, CTK 8-1, and CASA40-1 were produced the PHAs more than 80% of cell area. Isolate code CCA2-11 could produce the highest yield up to 85.77% in their cells (Table 4.9).

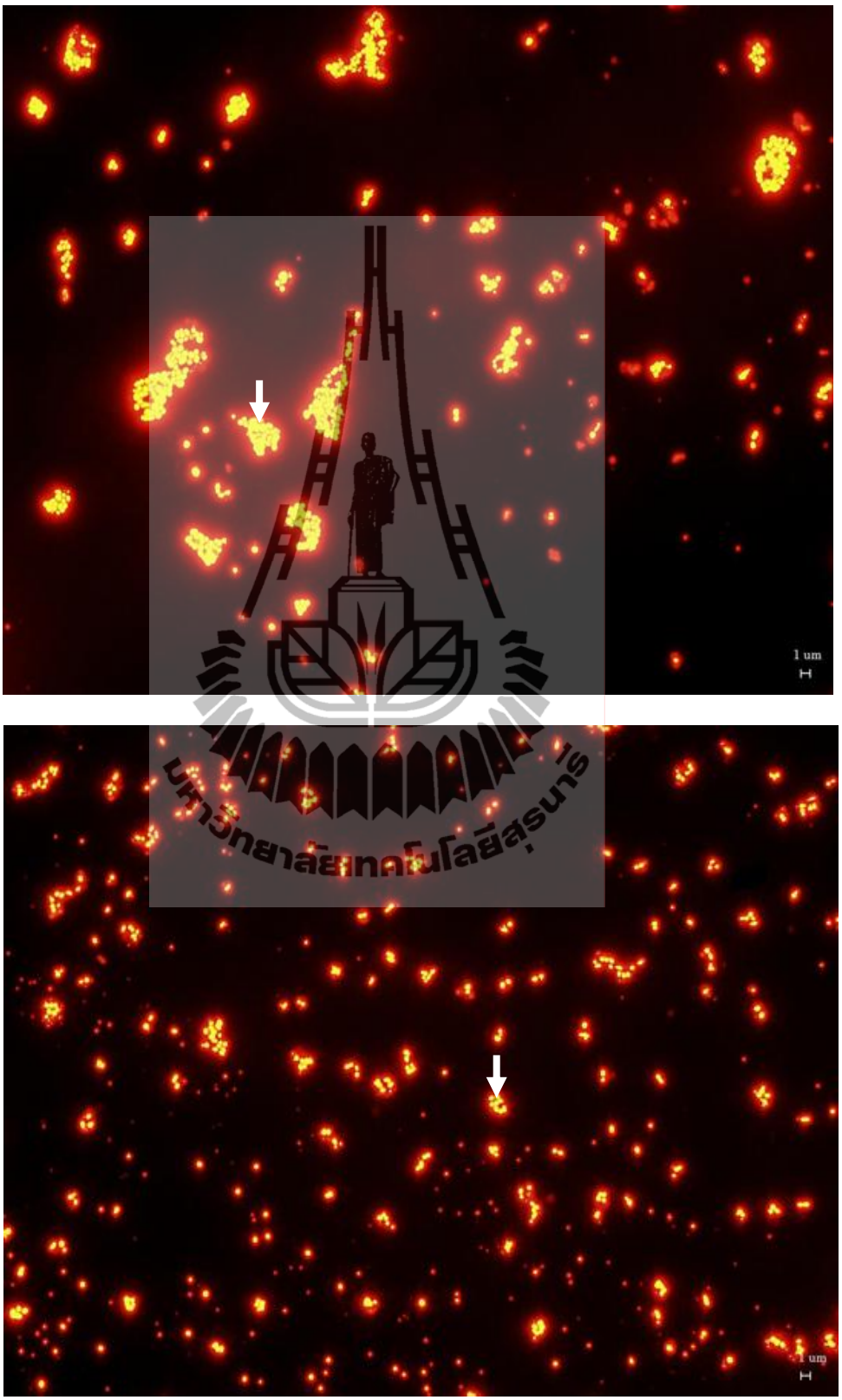


Figure 4.7 Bacterial cells accumulating PHAs stained with Nile blue A (arrows) after culturing on modified complex and minimal medium at 30°C for 48 h observed under fluorescence microscope.

Table 4.9 Percentage of PHA accumulation in cells of bacteria isolated from cassava pulp.

Bacterial group	No.	Isolate code	PHA accumulation (%)
Group 1 Gram-positive, rod shape, and non endospore-forming bacteria (15 isolates)	1	CWP 2-21	3.3
	2	CSP 2-6	5.5
	3	CTK 12	21.4
	4	CWP 2-15	22.1
	5	WCC2-8-1	22.21
	6	WCC2-15	31.19
	7	BPC2-12	32.47
	8	CAS 36	40.1
	9	PCA1-10-1	42.67
	10	WCC2-5	45.37
	11	CCA2-3	52.47
	12	BPC2-3	54.28
	13	CCS2-9	63.45
	14	BPC1-9	70.72
	15	CAS 5	74.0
Group 2 Gram-negative and rod shape bacteria (3 isolates)	16	CSP 2-3	5.1
	17	CSP 2-21	15.9
	18	CAS5-1	21.01
Group 3 Gram-positive, rod shape, and endospore-forming bacteria (59 isolates)	19	CST 2-44	8.3
	20	CWC 1-6-1	9.1
	21	CASA 51	11.2
	22	SC 4	13.7
	23	CSP 2-25-1-1	15.3
	24	CSP 2-25-2	16.1
	25	CACT 6	16.2
	26	CSP 2-23	16.9
	27	CSP 2-25	17.1
	27	CSP 2-25-1-1	17.4
	29	CCS1-14	17.81
	30	CASA 40	17.9
	31	CSP 2-26	18.9
	32	CAC 30	20.7
	33	CWC 1-6	20.8
	34	CAS 29	21.3
	35	CSP 1-18	22.1
	36	CTK 16	22.8
	37	CSP 2-27	23.4
	38	CST 2-45-2	23.8
	39	CSP 2-29-1	26.0
	40	CASA 48	26.6
	41	CASA 26	26.7

Table 4.9 (Continued) Percentage of PHA accumulation in cells of bacteria isolated from cassava pulp.

Bacterial group	No.	Isolate code	PHA accumulation (%)
(Continued) Group 3 Gram-positive, rod shape, and endospore-forming bacteria (59 isolates)	42	CASA51-1	27.1
	43	CASA 4	28.1
	44	CST 2-45-2	28.2
	45	CAT 8	28.4
	46	CSP 1-1	30.0
	47	CST 1-14	30.6
	48	CSC 2-2	32.0
	49	CSP 2-29-1-1-1	35.2
	50	CSP 1-4	36.2
	51	CAT 4	36.9
	52	CTK 8	38.9
	53	CSC 2-5	40.8
	54	CSP 2-22-1	41.7
	55	CSP 2-25-1	42.4
	56	CSP 2-22	43.7
	57	CASA 53-1	44.2
	58	CSP 2-7	47.5
	59	CSP 2-26-1	47.9
	60	CST 2-42-1-1	49.1
	61	CST 2-45-1	49.74
	62	CSC 1-37	49.9
	63	CASA 45-1	51.4
	64	CSC 2-3	53.5
	65	CSP 1-5	55.0
	66	CST 2-42-1	55.9
	67	CSP 2-25-1-2	58.2
	68	CASA 45	60.0
	69	CAC 24	60.7
	70	CSP 2-23-1	68.0
	71	CSP 2-29	73.1
	72	CWC 1-5	77.6
	73	CST 2-45-1-1	80.6
	74	CST 2-2	80.9
	75	CTK 8-1	81.7
	76	CASA 40-1	83.3
	77	CCA2-11	85.77
Group 4 Gram-positive, cocci shape, and non endospore-forming bacteria (5 isolates)	78	CWP2-16	51.9
	79	CCA1-24	56.67
	80	PCA1-13	57.95
	81	BCC2-7	73.22
	82	CCS1-13	74.99

4.2.2 Electron microscopes for the detection of PHAs-producing bacteria

PHA granules in bacterial cells could be detected using specific cultural media and staining with lipophilic dyes such as Nile blue, Nile red, or Sudan black B. However, fluorescence staining may cause false-positive result or over explosion of PHA granules in the bacterial cells. Precise and consistent detection methodology for bacterial PHA granules is still desired. Electron microscopy is one of the reliable detection methods. In this study, specimen preparation for the procedure for the efficient observation of bacterial PHA granules under transmission and scanning electron microscopes (TEM and SEM) were performed. The fixative solution composing of 5% glutaraldehyde, 1% OsO_4 and 0.2 M phosphate buffer was found to be suitable for this study when compared to the fixative solution (2% glutaraldehyde, 3% paraformaldehyde, 5% sucrose, and 0.1 M sodium cacodylate buffer, pH 7.4, and the secondary fixative solution was 1% osmium) (Tian *et al*., 2005). The suitable ratio of fixative solution was 1:1:1. Tactical of specimen's fixation was worked at 4°C. Acetone at series (20, 40, 60, 80, and 100%) gave better results than other organic solvents in the dehydration step when compared to ethanol series (50, 70, 95, and 100%) (Tian *et al*., 2005). Epon viscosity embedding resin with incubating at 60°C for 24 h was sufficient for TEM polymerization. After sectioning, copper grids were strained with uranyl acetate and lead acetate. This improved method could enhance the detection of PHA granules using electron microscopy (Figures 4.8-4.9).

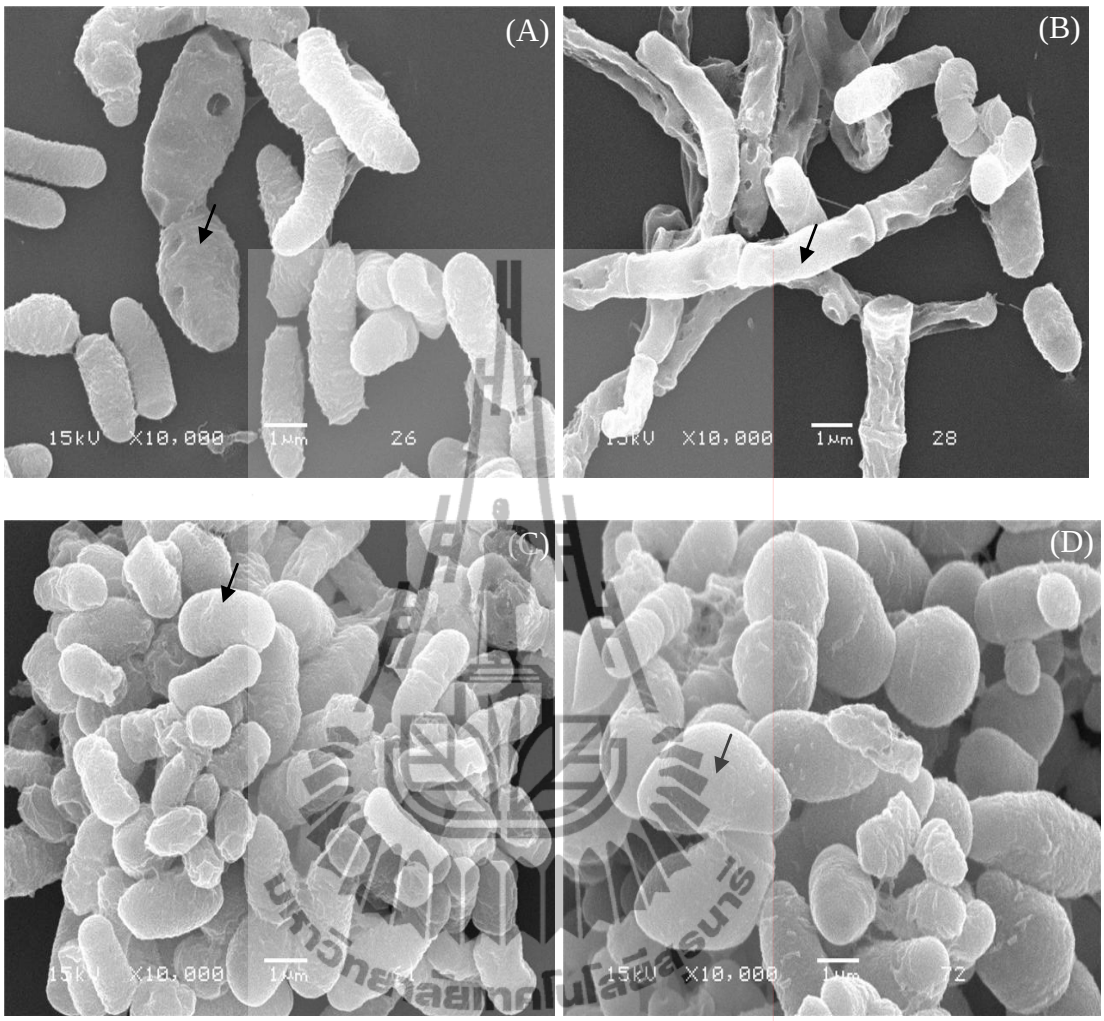


Figure 4.8 SEM micrographs of bacterial isolates CST2-2 (A), CASA40-1 (B), CST2-45-1-1 (C), and CCA2-11 (D) accumulating PHAs in their cells (arrows).

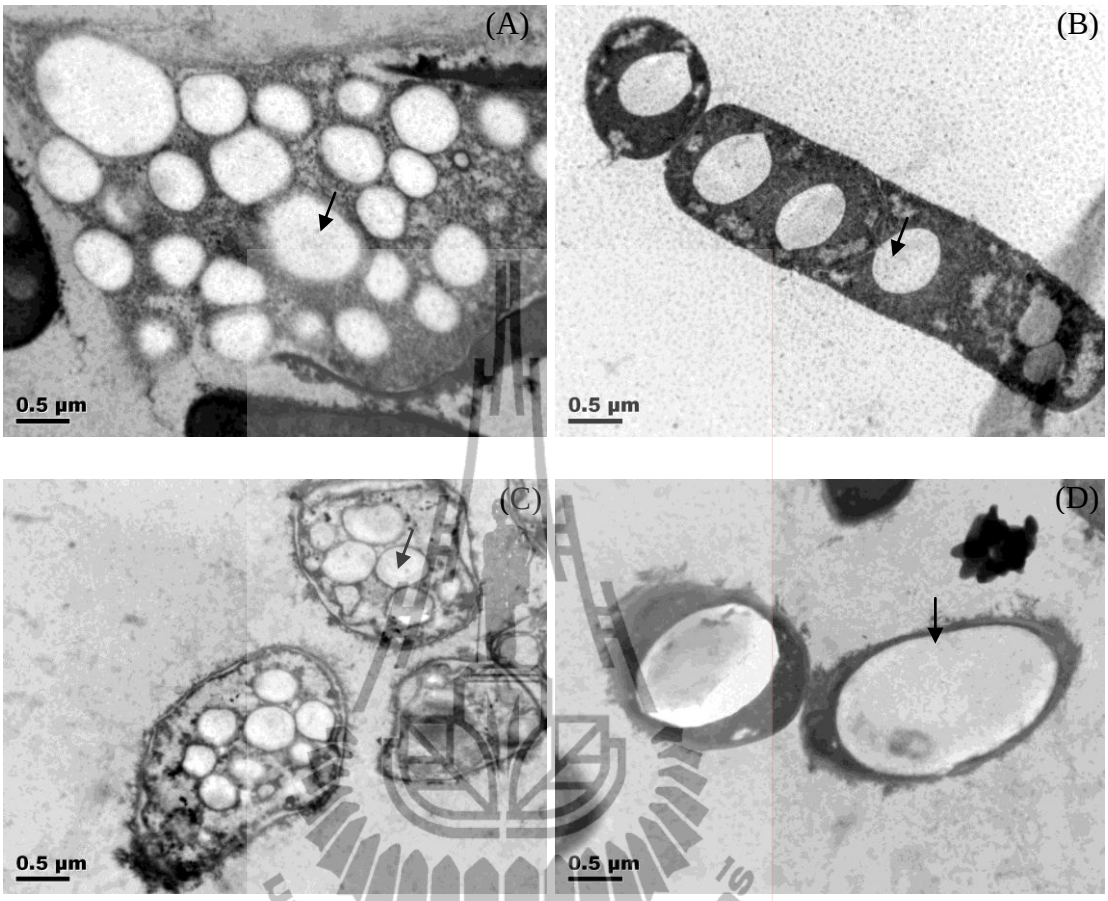


Figure 4.9 TEM micrographs of granules (arrows) in cells of bacterial isolates CST2-2 (A), CASA40-1 (B), CST2-45-1-1 (C), and CCA2-11 (D).

4.3 Monitoring of PHA accumulation in bacterial cells

Bacteria isolated from cassava pulp that found to accumulate PHAs in their cells were selected for monitoring PHA accumulation during cultivation using Nile red dyeing technique. The isolates were cultured in modified minimal broth supplemented with Nile red and incubated in an orbital shaker (100 rpm) at 30°C for 96 h. At 0, 24, 48, 72, and 96 h of cultivation, a 1-mL sample was removed, centrifuged, and washed the pellet, then re-suspended in 1 mL of 0.1 M glycine-HCl, incubating for 2 h, and measuring by spectrofluorophotometer at excitation and emission wavelengths 543 nm and 598 nm, respectively.

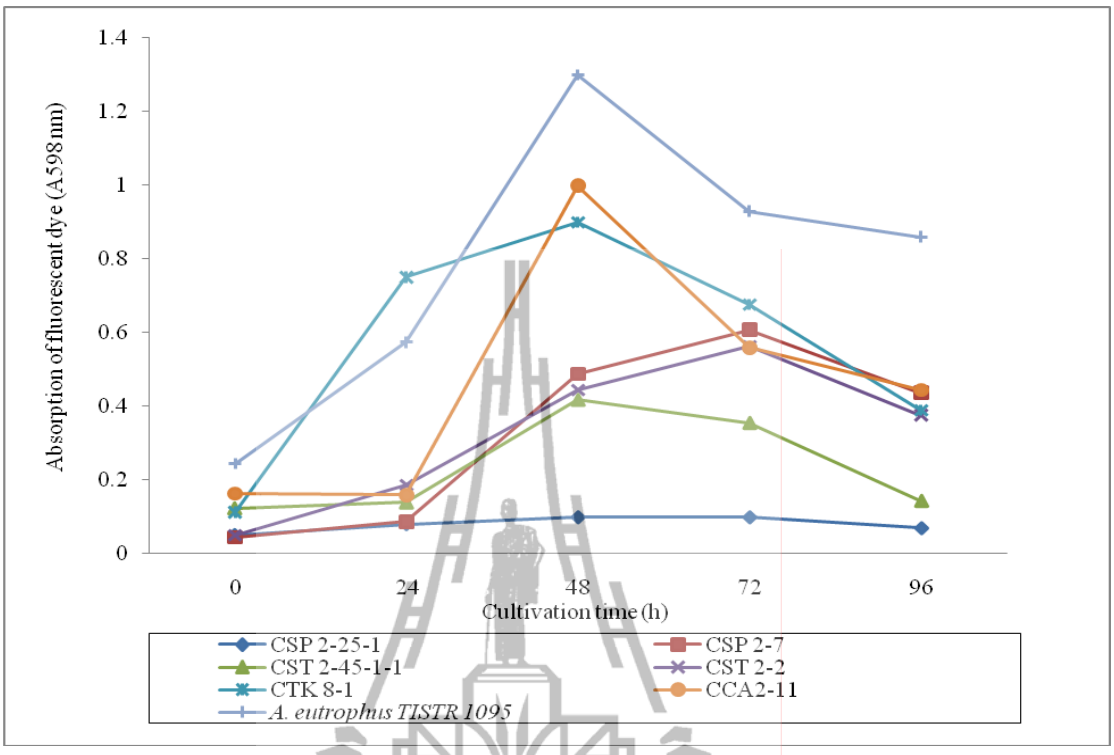


Figure 4.10 PHA accumulations in cells of Gram-positive, rod and endospore-forming bacteria using Nile red dyeing technique during cultivation at difference time. *A. eutrophus* TISTR 1095 was used as positive control.

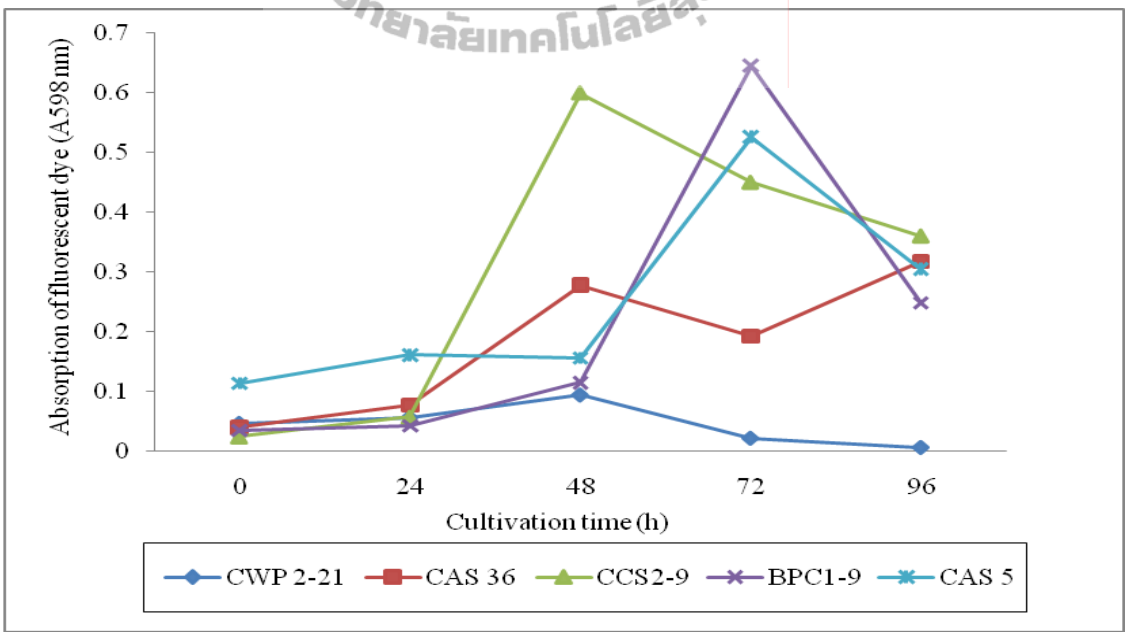


Figure 4.11 PHA accumulations in cells of Gram-positive, rod and non endospore-forming bacteria using Nile red dyeing technique during cultivation at difference time.

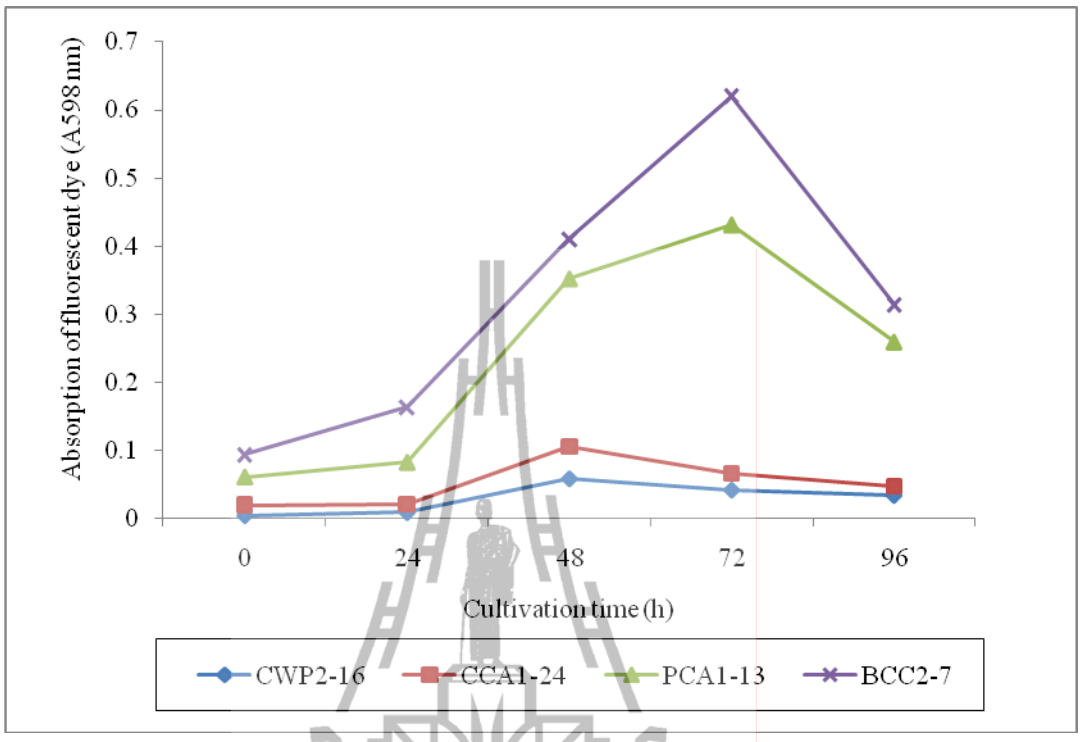


Figure 4.12 PHA accumulation in cells of Gram-positive, cocci and non endospore-forming bacteria using Nile red dyeing technique during cultivation at difference time.

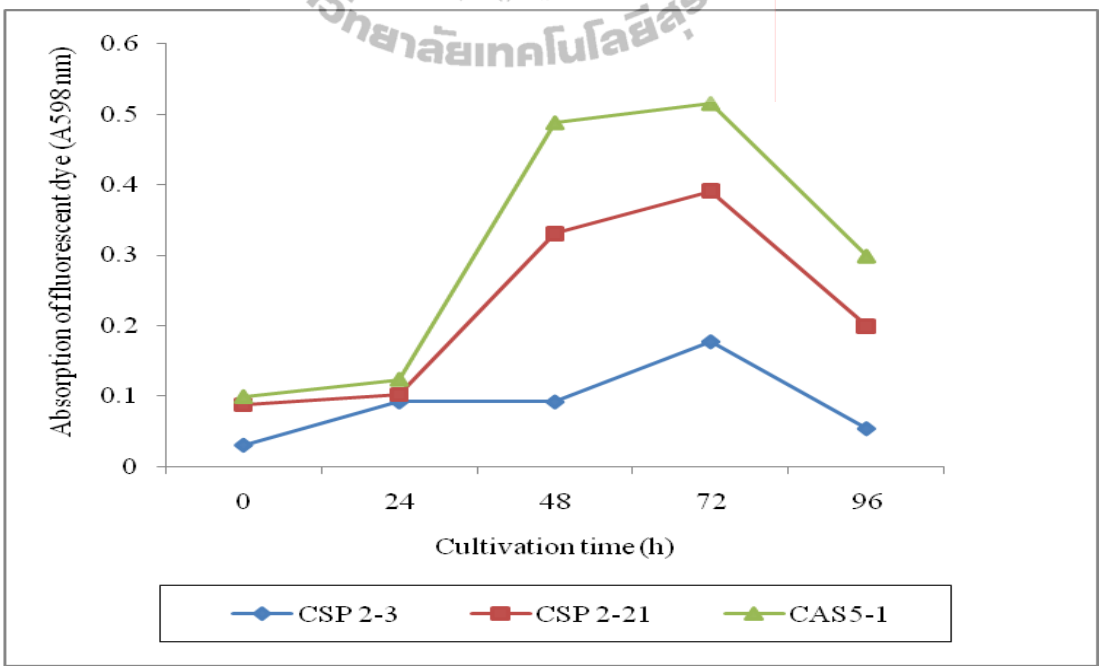


Figure 4.13 PHA accumulation in cells of Gram-negative, rod and non endospore-forming bacteria using Nile red dyeing technique during cultivation at difference time.

The bacteria could produce the maximum PHA yield at 48-72 h (Figures 4.10-4.13). PHA, the intracellular granules, formed under nutrient-limited. The polymers serve as reserves of carbon and reducing equivalents to preserve cell survival during stress conditions at incubation periods (Foster *et al*., 2005; Sudesh and Abe, 2010). In this study, at 48-72 h of incubation, bacteria produced the high concentration of PHAs. Then, when nutrient-limited, PHAs were used for energy reserve for cell survival. Concentration of PHAs decreased at 96 h of incubation. Berlanga *et al*. (2006) screened PHAs-producing bacteria isolated from microbial mats from Ebro Delta, Spain, and found the maximum fluorescence intensity of each strain when cultured in glucose and fructose medium was 48-72 h of incubation.

4.4 Identification of PHAs-producing bacteria

4.4.1 Morphological characterization

For morphological characterization of PHAs-producing bacteria, cell morphology of the bacteria, was examined. Four groups of bacteria according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) were concluded (Figure 4.14). Group 1 which was the regular, nonsporing Gram-positive rods comprised of 15 isolates. Groups 2-4 which were Gram-negative rod, endospore-forming Gram-positive rod, and Gram-positive cocci, respectively comprised of 3, 59, and 5 isolates (Table 4.10).

Table 4.10 Group of PHAs-producing bacteria isolated from cassava pulp according to their cell morphology.

Bacterial group	Cell size (μm)	Spore-forming	Amount of isolate
1. Gram-positive rods	0.3-0.5×2.0-2.4 to 0.5-0.8×2.2-3.1	_	15
2. Gram-negative rods	0.2-0.5×1.2-1.3 to 0.5-0.7×1.7-3.1	_	3
3. Gram-positive and spore-forming rods	0.4-0.7×1.1-1.4 to 0.77-1.05×3.67-6.38	+	59
4. Gram-positive cocci	0.18-0.28×0.18-0.28 to 0.4-1.2×0.4-1.2	_	5
			82

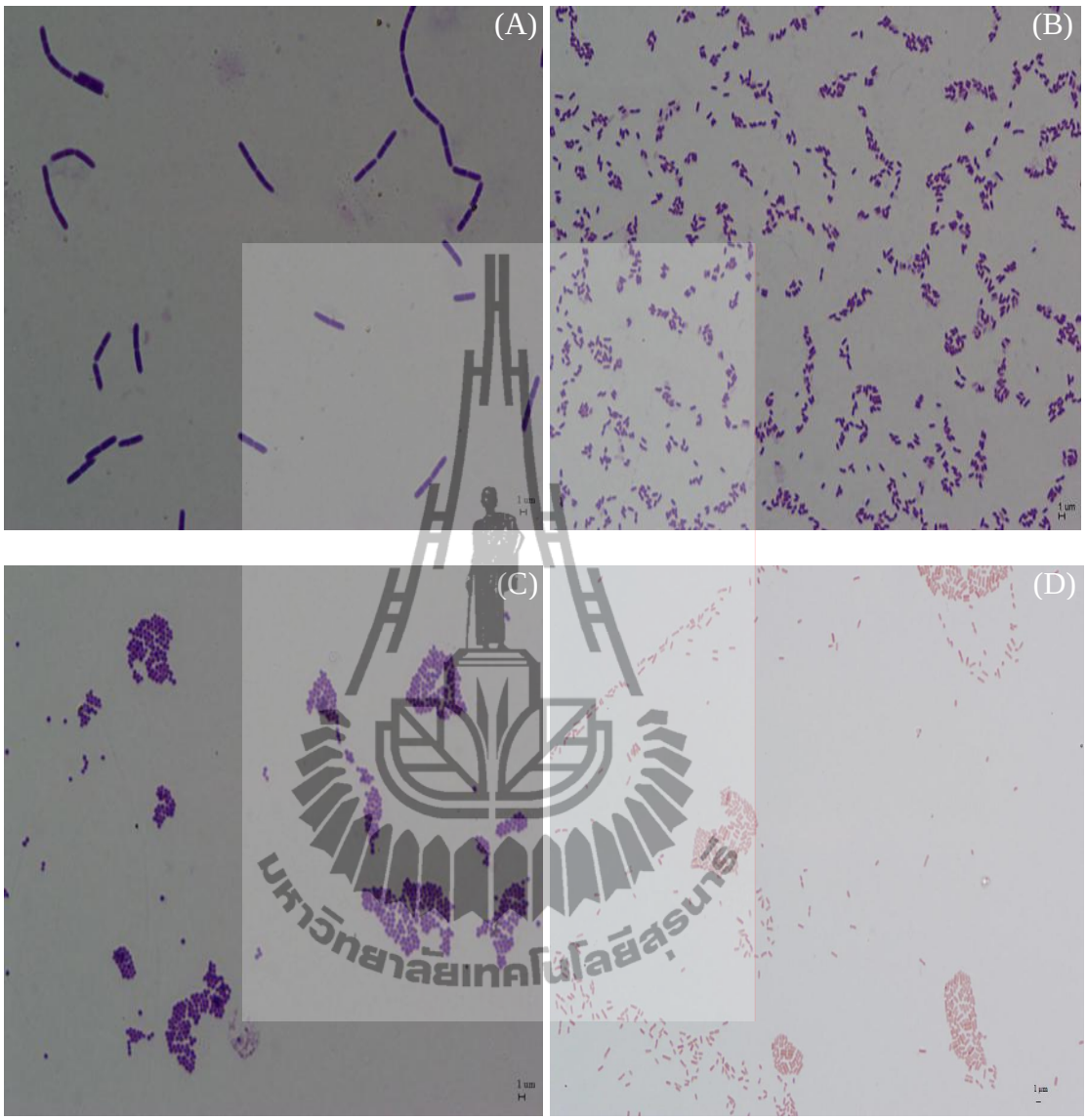


Figure 4.14 Gram-staining of Gram-positive, rod shape and spore-forming of isolate CSP2-23 (A), Gram-positive, rod shape and non spore-forming of isolate CAS36 (B), Gram-positive, cocci shape of isolate CWP2-16 (C), and Gram-negative, rod shape of isolate CSP2-21 (D), respectively.

4.4.2 Physiological characterization

PHAs-producing bacteria isolated from cassava pulp were found to have different physiological characteristics (Tables 4.10-4.11, and Figure 4.14). Results from API identification system (bioMérieux, Inc., France) (Figures 4.15-4.16 and Tables 4.12-4.15) were compared with known species in API online data base. Isolate PCA1-10-1 had 99.9% identity to *Listeria welshimeri*. Isolates CSP2-3, CSP2-21, and CAS5-1, had 99.7, 99.8, and 89.6% identity to *Chryseobacterium indologenes*. Isolates SC4, CASA51-1, CST2-2, CWC1-6-1, CSP2-25-1-1, CCA2-11, CSP2-25-1, CSC2-2, CSP2-23, and CSP2-26-1 had 99.9, 99.0, 99.4, 95.4, 99.7, 99.7, 97.9, 96.8, 98.8, and 93.1% identity to *Bacillus cereus*, *B. firmus*, *B. megaterium*, *B. mycoides*, *B. stearothermophilus*, *B. megaterium*, *B. cereus*, *B. cereus*, *B. stearothermophilus*, and *B. firmus*, respectively. Isolates CWP2-16, CCS1-13, BCC2-7, CCA1-24, and PCA1-13 had 99.6, 99.9, 85.7, 99.7, and 94.9 % identity to *Staphylococcus cohnii* subsp. *urealyticum*, *S. lentus*, *S. sciuri*, *Micrococcus* sp., and *S. xylosus*, respectively.

Table 4.11 Physiological characteristics of 13 bacterial isolates from cassava pulp.

Characteristics	Isolate No												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Gram reaction	+	+	+	+	+	+	+	+	+	+	-	-	-
Cell shape	R	R	R	R	R	C	C	C	C	C	R	R	R
Endospore-produced	+	+	+	+	+	-	-	-	-	-	-	-	-
Cell diameter > 1.0 µm	+	-	+	+	-	-	-	-	-	-	-	-	-
Spore round	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on NaCl agar													
10%	-	+	-	-	-	+	+	+	+	+	ND	ND	ND
15%	-	-	-	-	-	+	+	+	+	+	ND	ND	ND
Growth at (°C)													
5	-	-	+	-	-	ND	ND	ND	ND	ND	-	ND	ND
10	+	+	+	+	-	ND	ND	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	-	+	+	+	+	ND	ND	ND
30	+	+	+	+	-	+	+	+	+	+	+	+	+
40	+	+	+	+	+	ND	ND	ND	ND	ND	+	ND	ND
45	ND	ND	ND	ND	ND	-	-	-	-	ND	ND	ND	ND
50	-	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND
55	-	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND
65	-	-	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND
Motility	+	+	+	+	+	-	-	-	-	-	+	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	+	+	+	-	-	-	+	+	-	-
MR test	+	-	+	+	-	-	+	+	+	+	-	-	+
VP test	+	-	-	+	-	-	-	+	+	+	-	+	+
Indole test	-	-	-	-	-	-	-	-	-	-	-	+	-
Nitrate reaction test	+	-	-	+	-	+	+	+	-	-	+	+	+
Acid production from													
D-mannose	-	-	+	-	-	+	+	+	+	-	ND	+	+
Lactose	-	-	+	-	-	+	-	+	+	-	ND	+	+
D-fructose	+	+	+	+	+	+	+	+	+	-	ND	ND	+
D-galactose	-	-	+	+	-	+	+	+	-	-	ND	ND	+
D-xylose	-	-	+	-	+	-	-	+	-	-	ND	+	+
L-arabinose	-	-	+	-	+	+	+	+	-	-	ND	ND	+
D-manitol	-	+	+	-	+	+	+	+	+	-	ND	+	+
D-glucose	+	+	+	+	+	ND	ND	ND	+	-	+	+	+
Gelatin	+	+	+	+	+	-	-	-	-	+	+	-	+
Starch hydrolysis	+	+	+	+	+	-	+	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-

Symbols: - = negative; + = positive; C = cocci; ND = not detected; and R = rod

Figure 4.15 Pattern form API 50 CHB (bioMérieux, Inc., France) of bacterial isolates CWC1-6-1 (A), SC4 (B), CCA2-11 (C), CSP2-23 (D), CSP2-26-1 (E), and CSP2-25-1-1 (F) when incubated at 35°C for 48 h.

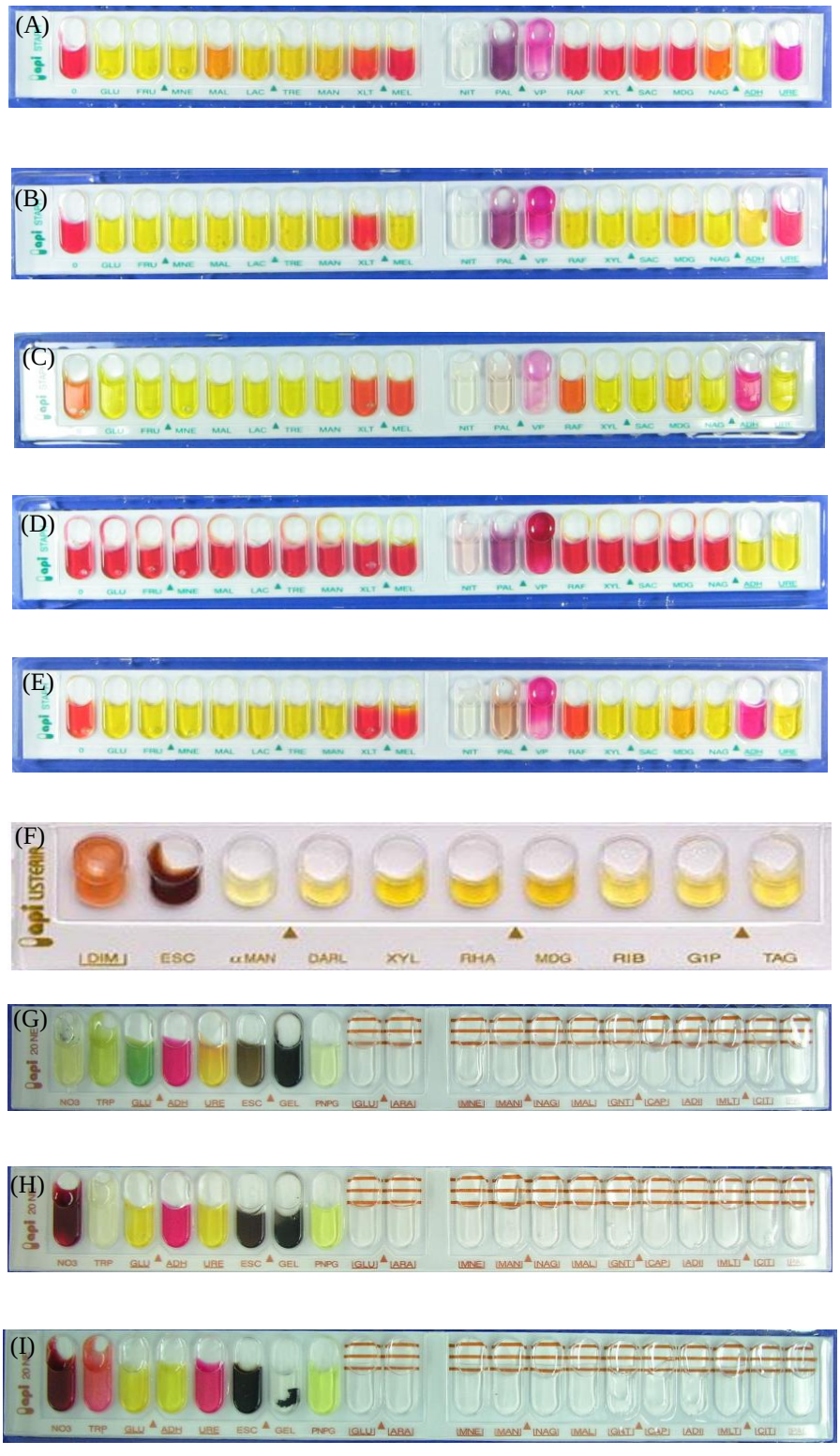


Figure 4.16 Patterns form API (bioMérieux, Inc., France), API Staph of bacterial isolates CWP2-16 (A), CSC1-13 (B), BCC2-7 (C), CCA1-24 (D), and PCA1-13 (E), API Listeria of bacterial isolate PCA1-10-1 (F), and API 20 NE of bacterial isolates CSP2-3 (G), CAS5-1 (H), and CSP2-21 (I) when incubated at 35°C for 24 h.

Table 4.12 Confirmation of biochemical test using API 50 CHB (bioMérieux, Inc., France).

Carbohydrate and some nutrient utilization	Selected isolate				
	CWC1-6-1	SC4	CCA2-11	CSP2-23	CSP2-26-1
Glycerol	-	+	+	-	-
Erythritol	-	-	-	-	-
D-arabinose	-	-	-	-	-
L-arabinose	-	-	+	-	-
D-ribose	+	+	+	+	-
D-xylose	-	-	+	-	-
L-xylose	-	-	-	-	-
D-adonitol	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-
D-galactose	-	-	-	-	-
D-glucose	+	+	+	+	+
D-fructose	+	+	+	+	+
D-mannose	+	+	-	-	-
L-sorbose	-	-	-	-	-
L-rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inositol	-	-	+	-	-
D-mannitol	-	-	+	-	-
D-sorbitol	-	-	+	-	-
Mehyl-αD-mannopyranoside	-	+	-	-	-
Mehyl-αD-glucopyranoside	-	+	-	-	-
N-acetylglucosamine	+	+	+	+	+
Amygdalin	-	+	+	-	-
Arbutin	-	+	+	-	-
Esculin ferric citrate	+	+	+	-	+
Salicin	+	+	+	-	-
D-cellobiose	-	+	+	-	-
D-maltose	+	+	+	+	+
D-lactose (bovine origin)	-	+	+	-	-
D-melibiose	-	-	+	-	-
D-saccharose (sucrose)	-	-	+	-	+
D-trehalose	+	+	+	+	+
Inulin	-	-	-	-	-
D-melezitose	-	-	-	-	-
D-raffinose	-	-	+	-	-
Amidon (starch)	+	+	+	+	-
Glycogen	+	-	+	+	-
Xylitol	-	+	-	-	-
Gentiobiose	-	-	+	-	-
D-turanose	-	-	-	-	-
D-lyxose	-	-	-	-	-
D-tagatose	-	-	-	-	-
D-fucose	-	-	-	-	-
L-fucose	-	-	-	-	-
D-arabitol	-	-	-	-	-
L-arabitol	-	-	-	-	-
Potassium gluconate	-	+	-	-	-
Potassium 2-ketogluconate	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	-

Table 4.13 Biochemical test results using API Staph (bioMérieux, Inc., France).

Carbohydrate and some nutrient utilization	Selected isolate				
	CWP2-16	CSC1-13	BCC2-7	CCA1-24	PCA1-13
D-glucose	+	+	+	-	+
D-fructose	+	+	+	-	+
D-mannose	+	+	+	-	+
D-maltose	+	+	+	-	+
D-lactose (bovine origin)	+	+	+	-	+
D-trehalose	+	+	+	-	+
D-mannitol	+	+	+	-	+
Xylitol	-	-	-	-	-
D-melibiose	-	+	-	-	-
Potassium nitrate	-	-	-	-	-
β-naphthyl phosphate	+	+	-	+	+
Sodium pyruvate	+	+	+	+	+
D-raffinose	-	+	-	-	-
D-xylose	-	+	+	-	+
D-saccharose (sucrose)	-	+	+	-	+
Mehyl-αD-glucopyranoside	-	+	+	-	+
N-acetylglucosamine	+	+	+	-	+
L-arginine	-	-	-	-	+
Urea	+	-	-	-	-

Table 4.14 Biochemical test results using API 20 NE (bioMérieux, Inc., France).

Carbohydrate and some nutrient utilization	Selected isolate		
	CSP2-3	CAS5-1	CSP2-21
Potassium nitrate	+	+	+
L-tryptophan	-	-	+
D-glucose	-	+	+
L-arginine	-	+	-
Urea	+	-	+
Esculin ferric citrate	+	-	+
Gelatin (bovine origin)	+	+	-
4-nitrophenyl-βD-galactopyranoside	+	+	+
D-glucose	+	-	-
L-arabinose	-	-	-
D-mannose	-	-	-
D-mannitol	-	-	-
N-acetylglucosamine	-	-	-
D-maltose	-	-	-
Potassium gluconate	-	-	-
Capric acid	-	-	-
Adipic acid	-	-	-
Malic acid	-	-	-
Trisodium citrate	-	-	-
Phenylacetic acid	-	-	-

Table 4.15 Biochemical test results using API Listeria (bioMérieux, Inc., France).

Carbohydrate and some nutrient utilization	Selected isolate CSP2-21
Enzymatic substrate	+
Esculin ferric citrate	+
4-nitrophenyl-βD-galactopyranoside	-
D-arabitol	+
D-xylose	+
L-rhamnose	-
Methyl-α-D-glucopyranoside	-
D-rhibose	+
Glucose-1-phosphate	+
D-tagatose	-

4.4.3 16S rRNA gene characterization

For 16S rRNA gene characterization of PHAs-producing isolates, genomic DNA was extracted from 12 bacterial isolates. 16S rDNA was amphified using primers fD1, and rP2. The size of amplified DNA fragment was about 1,500 bp. (Figure 4.17). Nucleotide sequence data were obtained (Appendix B). These 16S rDNA sequences were deposited into GenBank database with accession numbers of HQ179137 to HQ179145, and HQ179147 to HQ179149. Phylogenetic tree of these bacteria were contributed (Figures 4.18-4.22). These isolates had high similarity to type strains (Tables 4.16-4.20). When compared these sequences to sequences from GenBank database, it was found that 3 isolates of Gram-negative rods had 96.7, 99.1, and 99.5% similarity to *Pseudomonas aeruginosa* PAON 2, *Klebsiella oxytoca* SB 9, and *Enterobacter hormaechei* CIP 103441^{T}, respectively. Eight isolates of endospore-forming Gram-positive rods had 99.9, 99.2, 99.7, 98.0, 99.6, 99.2, 89.8, and 99.9% similarity to *Bacillus megaterium* ATCC 14581^{T}, *Bacillus cereus* ATCC 21281^{T}, *Bacillus* sp. NCCP-158^{T}, *B. cereus* ATCC 43881^{T}, *B. subtilis* KQC 85, *Bacillus* sp.

IMT 21^{T}, *B. cereus* JS-33, and *Bacillus* sp. PBCC 10^{T}, respectively. And a representative strain of Gram-positive cocci had 99.3% similarity to *Staphylococcus cohnii* ATCC 29974^{T}. Identification results of selected PHAs-producing bacterial isolates based on API identification system (bioMérieux, Inc., France) compared to 16S rRNA gene sequence and similarity of 16S rRNA gene sequence of selected PHAs-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI) were concluded in Tables 4.21-4.22.

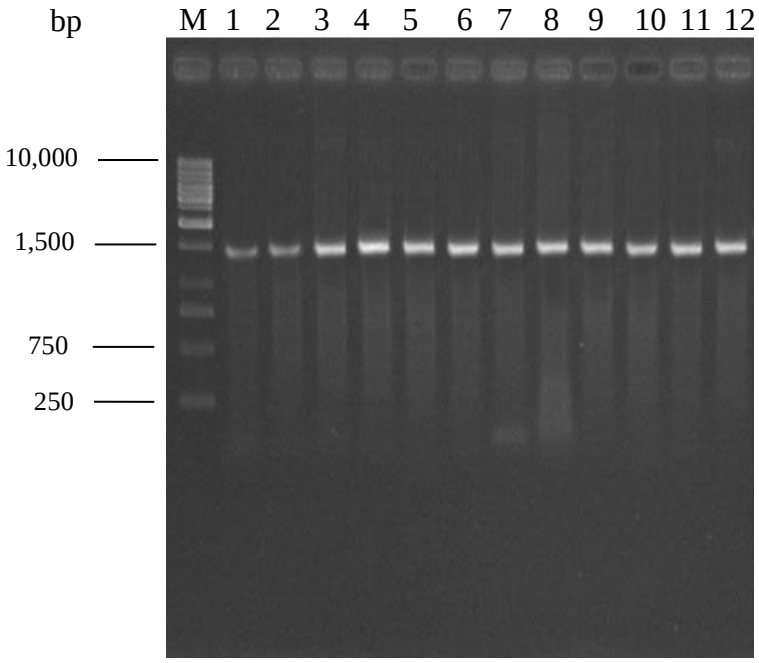


Figure 4.17 Gel electrophoresis of PCR products obtained from the amplification of bacterial 16S rDNA using primers fD1and rP2. Lanes: M, Marker 1 kb; 1 to 12 were strains SC4, CST2-2, CCA2-11, CSP2-25-1, CCA-24, CSP2-25-1-1, CAS5-1, CSP2-21, CWP2-16, CWP1-6-1, CASA51-1, and CSP2-3, respectively.

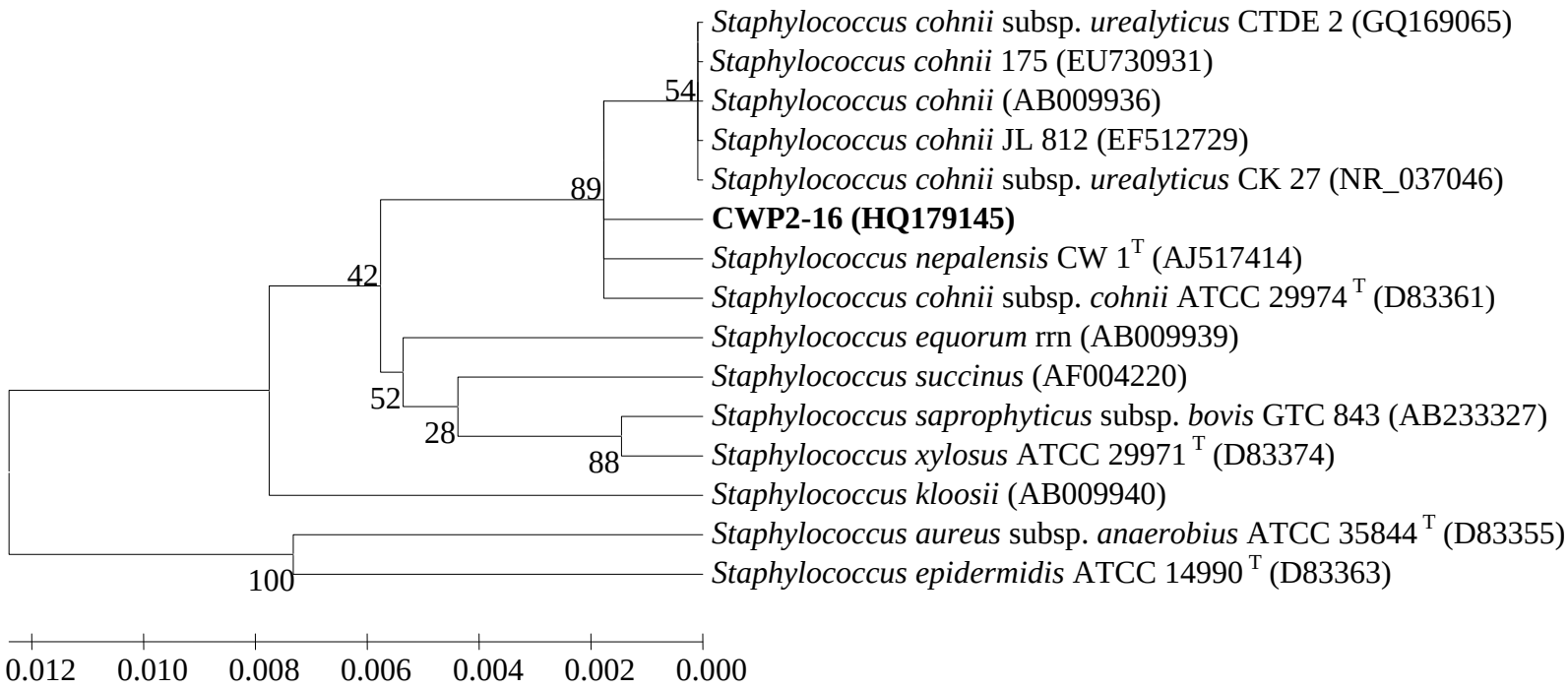


Figure 4.18 Phylogenetic tree of PHAs-producing Gram-positive cocci, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

Table 4.16 16S rRNA gene sequence similarity of Gram-positive cocci and related species.

Bacterial isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	100														
2	99.1	100													
3	99.5	99.5	100												
4	99.5	99.5	100	100											
5	99.4	99.4	99.9	99.9	100										
6	99.5	99.5	100	100	99.9	100									
7	99.5	99.5	100	100	99.9	100	100								
8	99.3	99.3	99.7	99.7	99.7	99.7	99.7	100							
9	98.5	98.4	98.9	98.9	98.8	98.9	98.9	98.9	100						
10	98.5	98.6	98.9	98.9	98.8	98.9	98.9	98.6	98.9	100					
11	98.6	98.6	99	99	98.9	99	99	98.8	98.8	98.9	100				
12	98.6	98.6	99	99	98.9	99	99	98.8	98.9	99.2	99.7	100			
13	98.2	98.2	98.6	98.6	98.6	98.6	98.6	98.4	98	98.6	98.1	98.3	100		
14	97.3	97.4	97.7	97.7	97.6	97.7	97.7	97.5	97.3	97.4	97.6	97.7	97.1	100	
15	97.3	97.6	97.7	97.7	97.6	97.7	97.7	97.6	97	97.3	97.6	97.5	97.3	98.5	100

1: CWP2-16 (HQ179145), 2: *Staphylococcus nepalensis* CW 1^T (AJ517414), 3: *S. cohnii* JL 812 (EF512729), 4: *S. cohnii* subsp. *urealyticus* CTDE 2 (GQ169065), 5: *S. cohnii* 175 (EU730931), 6: *S.cohnii* (AB009936), 7: *S. cohnii* subsp. *urealyticus* CK 27 (NR_037046), 8: *S. cohnii* subsp. *cohnii* ATCC 29974 ^{T} (D83361), 9: *S. equorum* rrn (AB009939),10: *S. succinus* (AF004220), 11: *S. saprophyticus* subsp. *bovis* GTC 843 (AB233327), 12: *S. xylosus* ATCC 29971^T (D83374), 13: *S. kloosii* (AB009940), 14: *S. aureus* subsp. *anaerobius* ATCC 35844 ^{T} (D83355), and 15: *S. epidermidis* ATCC 14990 ^{T} (D83363).

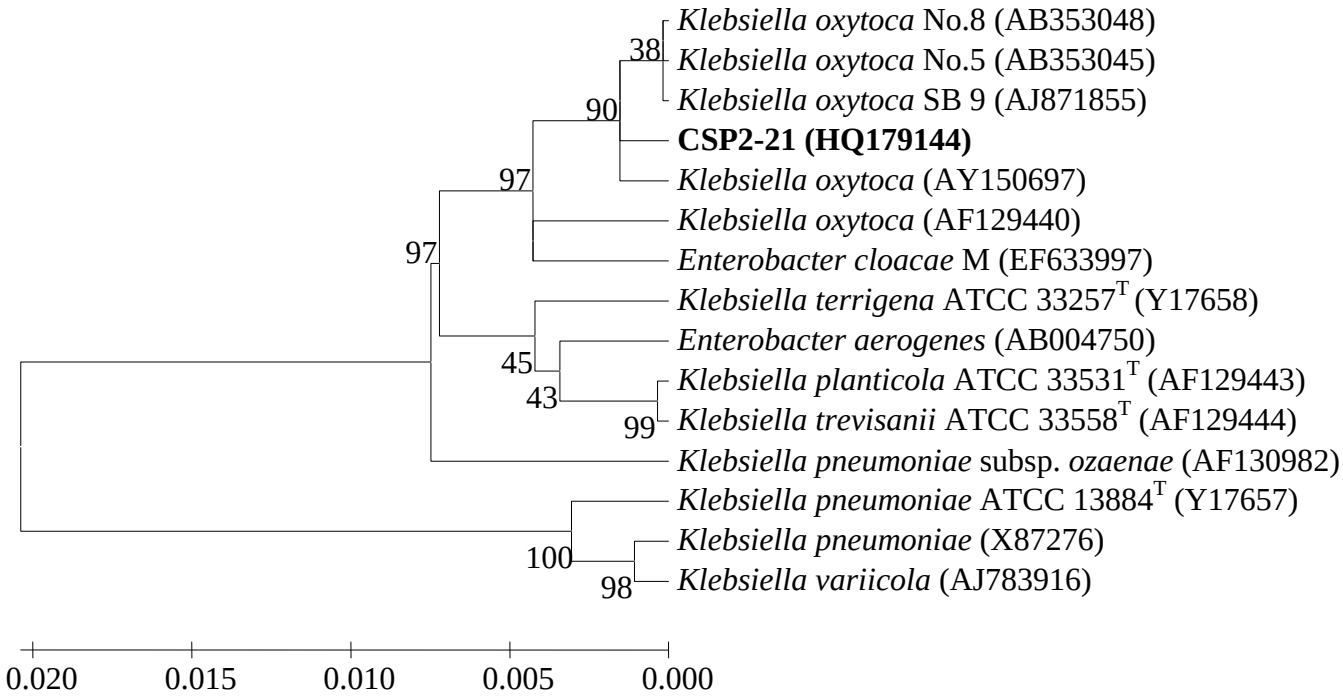


Figure 4.19 Phylogenetic tree of PHAs-producing Gram-negative rods, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

Table 4.17 16S rRNA gene sequence similarity of Gram-negative rods and related species.

Bacterial isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	100														
2	97.8	100													
3	97.8	99.8	100												
4	97.7	98.5	98.5	100											
5	98	99.2	99.2	99.2	100										
6	97.9	99	99	98.4	99.2	100									
7	98.9	98.5	98.7	98.4	98.7	98.8	100								
8	98.1	97.9	97.9	97.7	97.9	98.3	98.9	100							
9	99.1	98.7	98.7	98.6	98.9	98.8	99.8	98.9	100						
10	99.1	98.7	98.7	98.6	98.9	98.8	99.8	98.9	100	100					
11	98.9	98.5	98.6	98.6	98.7	98.7	99.7	98.9	99.8	99.8	100				
12	98.7	98.6	98.6	98.5	98.8	98.7	99.4	98.9	99.6	99.6	99.4	100			
13	95.3	95.7	95.7	96.8	96.2	95.7	95.9	95.3	95.9	95.9	95.9	95.7	100		
14	95.2	95.7	95.7	96.7	96.1	95.7	95.8	95.4	95.8	95.8	95.9	95.7	99.7	100	
15	95.8	96	96.2	97.3	96.7	96.1	96.4	95.5	96.4	96.4	96.4	96	99.4	99.2	100

1: CSP2-21 (HQ179144), 2: *Klebsiella planticola* ATCC 33531^{T} (AF129443), 3: *K. trevisanii* ATCC 33558^{T} (AF129444), 4: *K. pneumoniae* subsp. *ozaenae* (AF130982), 5: *Enterobacter aerogenes* (AB004750), 6: *K.terrigena* ATCC 33257^{T} (Y17658), 7: *K. oxytoca* No.8 (AB353048), 8: *K. oxytoca* (AF129440), 9: *K.oxytoca* No.5 (AB353045), 10: *K. oxytoca* SB 9 (AJ871855), 11: *K. oxytoca* (AY150697), 12: *E. cloacae* M (EF633997), 13: *K.pneumoniae* (X87276), 14: *K. variicola* (AJ783916), and 15: *K. pneumoniae* ATCC 13884^{T} (Y17657).

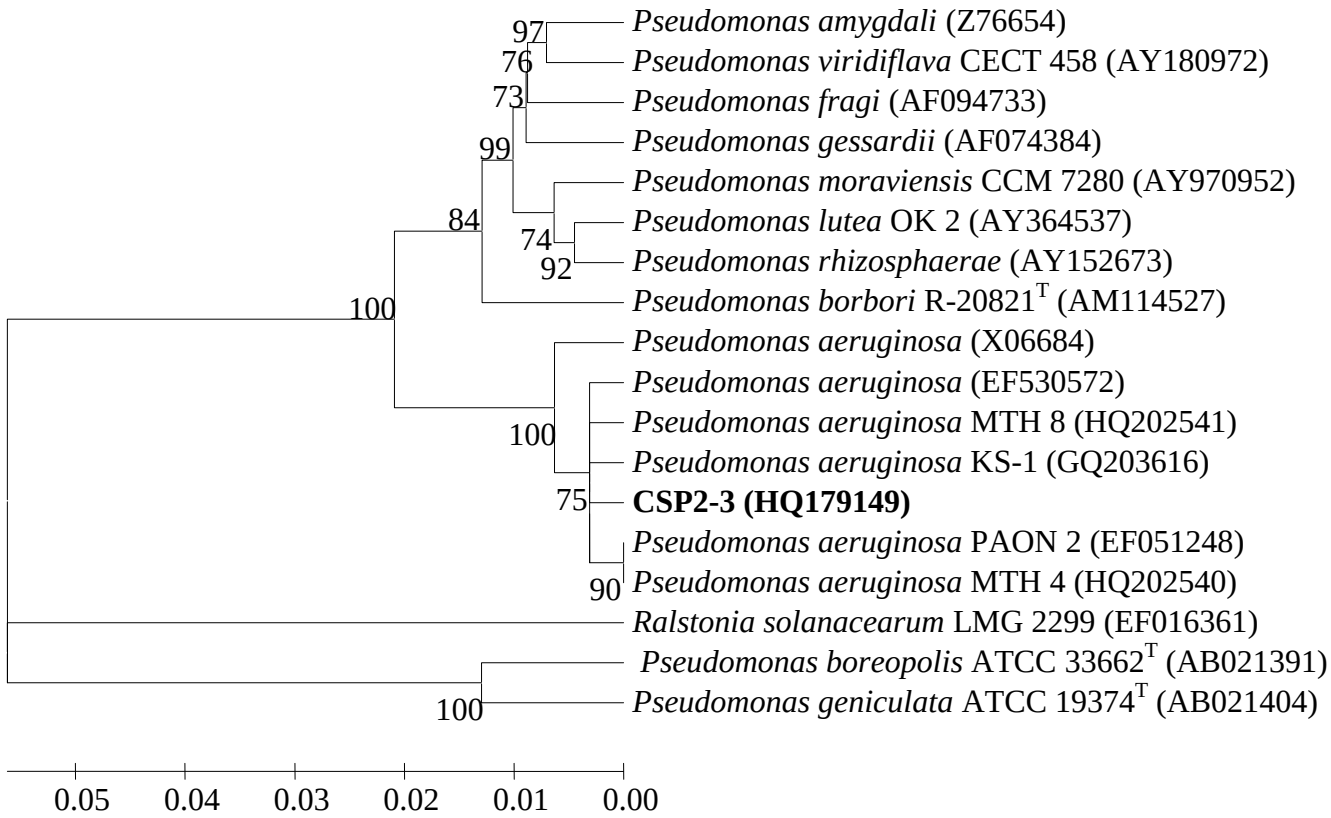


Figure 4.20 Phylogenetic tree of PHAs-producing Gram-negative rods, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

Table 4.18 16S rRNA gene sequence similarity of Gram-negative rods and related species.

Bacterial isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	100																	
2	96.5	100																
3	96.7	99.4	100															
4	96.7	99.4	100	100														
5	96.5	99.2	99.7	99.7	100													
6	96.6	99.1	99.7	99.7	99.9	100												
7	95.6	97.9	98.4	98.4	98.7	98.7	100											
8	91.6	94.1	94.5	94.5	94.7	94.6	93.8	100										
9	91.4	93.7	94.1	94.1	94.4	94.4	93.6	98.5	100									
10	91.7	94.1	94.6	94.6	94.8	94.7	93.8	98.2	97.7	100								
11	90.8	92.8	93.2	93.2	93.4	93.3	92.6	96.4	96.4	96.5	100							
12	90.7	92.8	93.2	93.2	93.4	93.5	92.6	96.9	97.1	96.2	97.7	100						
13	91.4	93.5	93.9	93.9	94.1	94.2	93.3	96.6	97.1	96.9	96.9	97.4	100					
14	91.5	93.6	94	94	94.2	94.3	93.4	96.8	96.7	97.7	96.6	97.2	97.7	100				
15	92.7	94.9	95.3	95.3	95.5	95.6	94.4	95.6	95.8	95.6	95.3	96.4	96.6	96.5	100			
16	82.9	84.8	85.2	85.2	85.4	85.4	84.9	85.9	85.6	85.9	85.6	85.2	86	86.5	85.8	100		
17	82.9	84.9	85.2	85.2	85.4	85.4	84.7	85.8	85.4	85.9	85.5	85.2	86.1	86.5	86.6	95.9	100	
18	81.0	82.8	83.1	83.1	83.3	83.3	82.7	82.6	82.6	82.5	81.9	81.9	82.3	82.8	82.5	83.9	84.5	100

1: CSP2-3 (HQ179149), 2: *Pseudomonas aeruginosa* KS-1 (GQ203616), 3: *P. aeruginosa* PAON 2 (EF051248), 4: *P. aeruginosa* MTH 4 (HQ202540), 5: *P. aeruginosa* MTH 8 (HQ202541), 6: *P. aeruginosa* (EF530572), 7: *P. aeruginosa* (X06684), 8: *P. lutea* OK 2 (AY364537), 9: *P. rhizosphaerae* (AY152673), 10: *P. moraviensis* CCM 7280 (AY970952), 11: *P.amygdali* (Z76654), 12: *P. viridiflava* CECT 458 (AY180972), 13: *P. fragi* (AF094733), 14: *P. gessardii* (AF074384), 15: *P. borbori* R-20821^{T} (AM114527), 16: *P. boreopolis* ATCC 33662^{T} (AB021391), 17: *P. geniculata* ATCC 19374^{T} (AB021404), and 18: *Ralstonia solanacearum* LMG 2299 (EF016361).

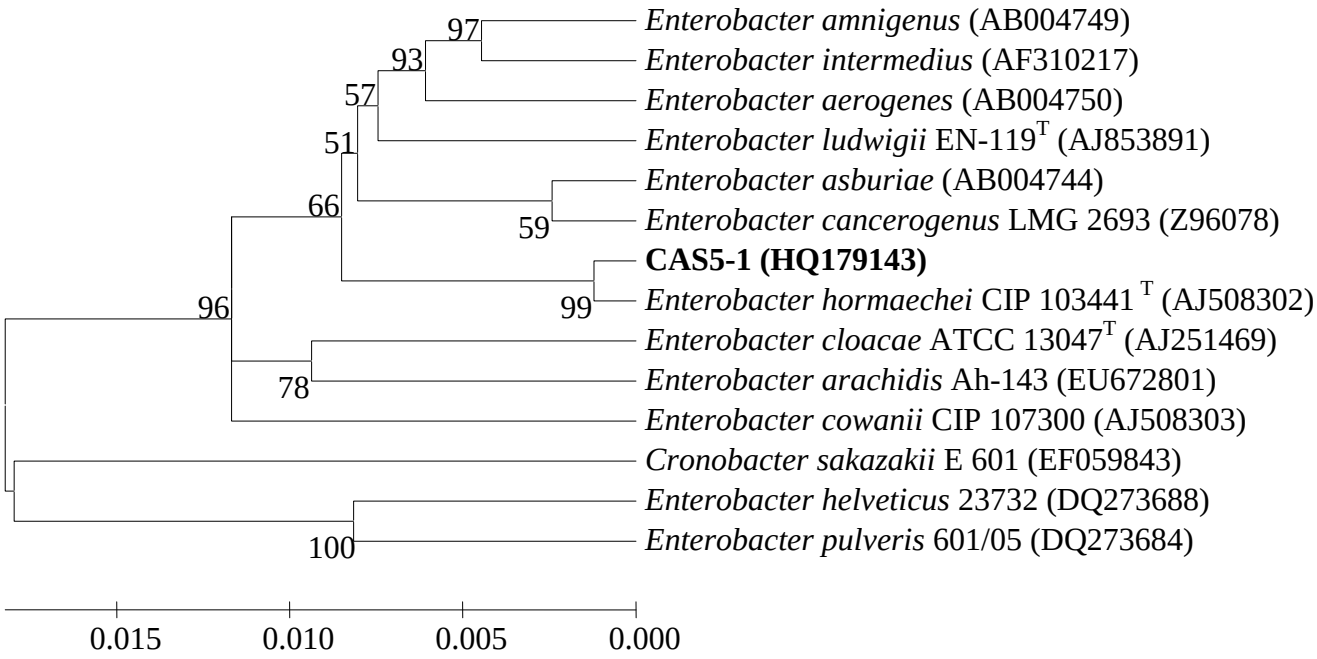


Figure 4.21 Phylogenetic tree of PHAs-producing Gram-negative rods, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

Table 4.19 16S rRNA gene sequence similarity of Gram-negative rods and related species.

Bacterial isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	100													
2	97.4	100												
3	97.5	99	100											
4	97.6	98.7	98.7	100										
5	98.7	97.7	97.5	98.6	100									
6	98.2	98.1	97.8	99	99.4	100								
7	98.6	98.3	98.2	98.5	98.6	99.1	100							
8	98.3	97.3	97.1	97.5	98.2	98	98.7	100						
9	96.4	96.1	95.9	96.7	97.4	97.4	97.1	97.9	100					
10	99.5	97.7	97.8	97.9	98.7	98.5	98.8	98.1	96.6	100				
11	98.2	96.5	96.5	96.8	98.2	97.7	97.5	98.1	97.3	98.2	100			
12	96.7	95.6	95.8	95.9	96.1	96.2	96.9	96.8	95.4	96.7	96.7	100		
13	96	96.5	96.3	96.8	96.4	96.7	96.5	96.3	95.8	96.2	96.3	96.3	100	
14	96.2	95.7	95.5	96.3	96.7	96.5	96.3	96.7	95.9	96.2	96.5	96.3	98.3	100

1: CAS5-1 (HQ179143), 2: *Enterobacter amnigenus* (AB004749), 3: *E. intermedius* (AF310217), 4: *E. aerogenes* (AB004750), 5: *E.asburiae* (AB004744), 6: *E.cancerogenus* LMG 2693 (Z96078), 7: *E.ludwigii* EN-119^{T} (AJ853891), 8: *E. cloacae* ATCC 13047^{T} (AJ251469), 9: *E. arachidis* Ah-143 (EU672801), 10: *E.hormaechei* CIP 103441 ^{T} (AJ508302), 11: *E. cowanii* CIP 107300^{T} (AJ508303), 12: *Cronobacter sakazakii* E 601 (EF059843), 13: *E. helveticus* 23732 (DQ273688), and 14: *E. pulveris* 601/05 (DQ273684).

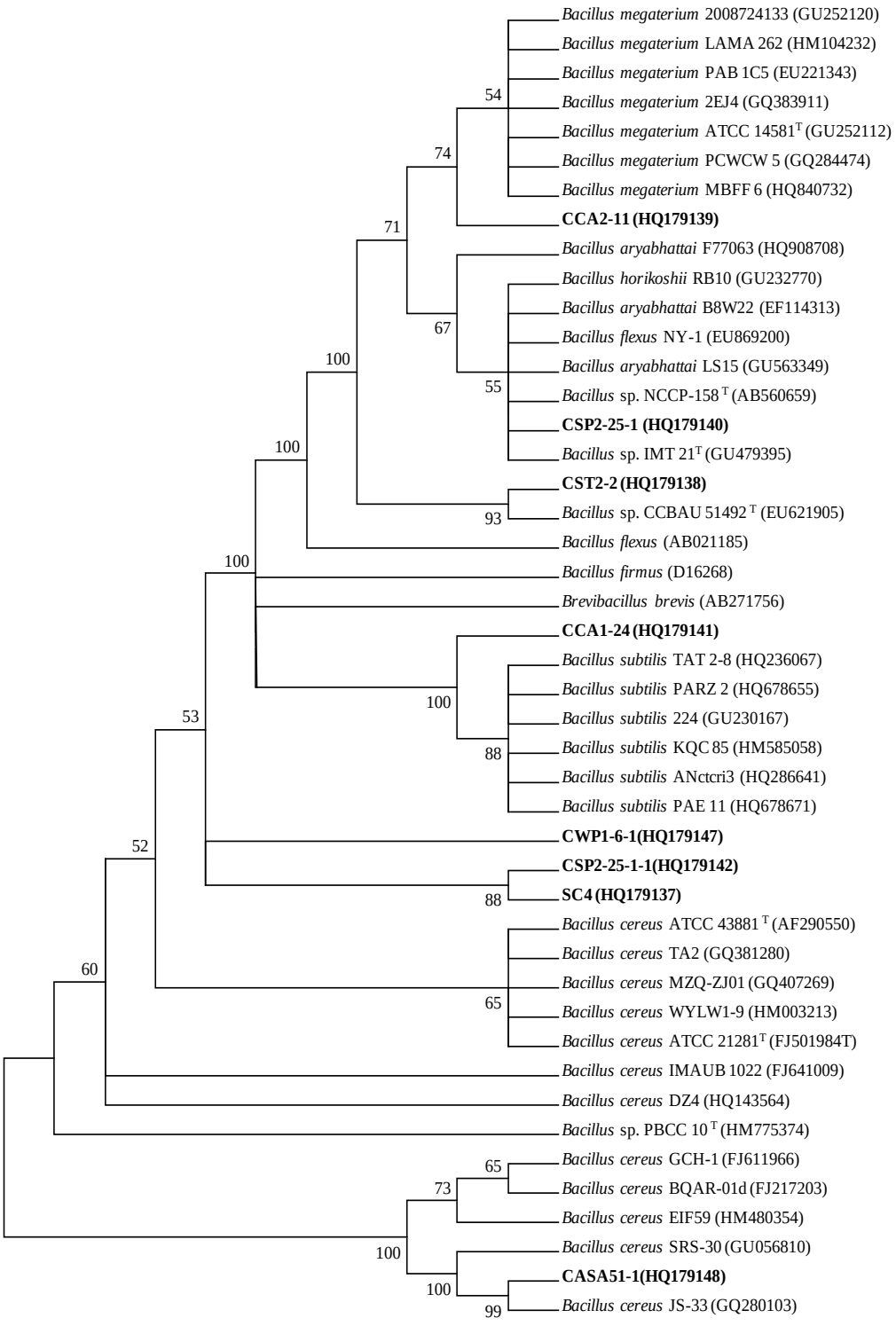


Figure 4.22 Phylogenetic tree of PHAs-producing Gram-positive rods endospore-forming, based on 16S rRNA gene sequence data using the neighbour-joining method. The unrooted tree was derived by using Clustal X, BioEdit, and Mega 4 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications.

Table 4.20 16S rRNA gene sequence similarity of Gram-positive rod endospore-forming and related species.

Bacterial isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	100																				
2	98.4	100																			
3	99.5	98.3	100																		
4	99.9	98.4	99.6	100																	
5	99.7	98.5	99.7	99.8	100																
6	99.7	98.5	99.7	99.7	99.9	100															
7	99.7	98.5	99.7	99.8	100	99.9	100														
8	99.7	98.5	99.7	99.8	100	99.9	100	100													
9	99.7	98.5	99.7	99.8	100	99.9	100	100	100												
10	99.7	98.5	99.7	99.8	100	99.9	100	100	100	100											
11	99.7	98.5	99.7	99.8	100	99.9	100	100	100	100	100										
12	99.9	98.4	99.6	100	99.8	99.7	99.8	99.8	99.8	99.8	99.8	100									
13	99.9	98.4	99.6	100	99.8	99.7	99.8	99.8	99.8	99.8	99.8	100	100								
14	99.9	98.4	99.6	100	99.8	99.7	99.8	99.8	99.8	99.8	99.8	100	100	100							
15	99.9	98.4	99.6	100	99.8	99.7	99.8	99.8	99.8	99.8	99.8	100	100	100	100						
16	99.9	98.4	99.6	100	99.8	99.7	99.8	99.8	99.8	99.8	99.8	100	100	100	100	100					
17	99.9	98.4	99.6	100	99.8	99.7	99.8	99.8	99.8	99.8	99.8	100	100	100	100	100	100				
18	99	98.5	99	99.1	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.1	99.1	99.1	99.1	99.1	99.1	100			
19	98.8	97.7	98.5	98.9	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.9	98.9	98.9	98.9	98.9	98.9	98.2	100		
20	88.9	88	88.8	89	89	89	89	89	89	89	89	89	89	89	89	89	89	88.5	89	100	
21	95	94	94.7	94.9	95	94.9	95	95	95	95	95	94.9	94.9	94.9	94.9	94.9	94.9	94.5	95.7	88.5	100

1: CCA2-11 (HQ179139), 2: CST2-2 (HQ179138), 3: CSP2-25-1 (HQ179140), 4: *Bacillus megaterium* PCWCW5 (GQ284474), 5: *B. aryabhattai* LS15 (GU563349), 6: *B. aryabhattai* F77063(HQ908708), 7: *B. flexus* NY-1 (EU869200), 8: *Bacillus sp.* IMT 21^{T} (GU479395), 9: *Bacillus sp.* NCCP-158^{T} (AB560659), 10: *B. aryabhattai* B8W22 (EF114313), 11: *B. horikoshii* RB10 (GU232770), 12: *B. megaterium* 2008724133 (GU252120), 13: *B. megaterium* PAB1C5 (EU221343), 14: *B. megaterium* MBFF6 (HQ840732), 15: *B.*

megaterium 2EJ4 (GQ383911), 16: *B. megaterium* ATCC 14581^{T} (GU252112), 17: *B. megaterium* LAMA 262 (HM104232), 18: *Bacillus sp.* CCBAU 51492^{T} (EU621905), 19: *B. flexus* (AB021185), 20: *Brevibacillus brevis* (AB271756), and 21: *B. firmus* (D16268).

Table 4.20 (Contonued) 16S rRNA gene sequence similarity of Gram-positive rod endospore-forming and related species.

Bacterial isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	100																							
2	93.5	100																						
3	91.9	97.7	100																					
4	92.9	98.5	98.2	100																				
5	81.9	86.7	86.5	87	100																			
6	99.6	93.1	92.1	93.1	82.2	100																		
7	99.6	93.1	92.1	93.1	82.2	100	100																	
8	99.6	93.1	92.1	93.1	82.2	100	100	100																
9	99.6	93.1	92.1	93.1	82.2	100	100	100	100															
10	99.6	93.1	92.1	93.1	82.2	100	100	100	100	100														
11	99.6	93.1	92.1	93.1	82.2	100	100	100	100	100	100													
12	93.5	99	98	99.2	87	93.6	93.6	93.6	93.6	93.6	93.6	100												
13	93.5	99	98	99.2	87	93.6	93.6	93.6	93.6	93.6	93.6	100	100											
14	93.7	99.1	98	99	87.1	93.7	93.7	93.7	93.7	93.7	93.7	99.7	99.7	100										
15	93.7	99.1	98	99	87.1	93.7	93.7	93.7	93.7	93.7	93.7	99.7	99.7	100	100									
16	93.5	99	98	99.2	87	93.6	93.6	93.6	93.6	93.6	93.6	100	100	99.7	99.7	100								
17	93.5	99	98	99.2	87	93.6	93.6	93.6	93.6	93.6	93.6	100	100	99.7	99.7	100	100							
18	93.5	99	98	99.2	87	93.6	93.6	93.6	93.6	93.6	93.6	100	100	99.7	99.7	100	100	100						
19	93.7	99	98	99.1	87.2	93.7	93.7	93.7	93.7	93.7	93.7	99.8	99.8	99.9	99.9	99.8	99.8	99.8	100					
20	91.5	96.9	95.7	96.8	86.8	91.6	91.6	91.6	91.6	91.6	91.6	97.5	97.5	97.7	97.7	97.5	97.5	97.5	97.7	100				
21	91.2	96.6	95.6	96.7	87	91.3	91.3	91.3	91.3	91.3	91.3	97.4	97.4	97.5	97.5	97.4	97.4	97.4	97.5	98	100			
22	86.2	91.5	90.5	91.5	84.1	86.2	86.2	86.2	86.2	86.2	86.2	92.2	92.2	92.4	92.4	92.2	92.2	92.2	92.3	93.9	94	100		
23	86	91.2	90.3	91.2	88.2	86.1	86.1	86.1	86.1	86.1	86.1	92	92	92	92	92	92	92	92.1	91.4	91.7	88.2	100	
24	82.2	87.4	87.1	87.7	89.8	82.5	82.5	82.5	82.5	82.5	82.5	88	88	88	88	88	88	88	88.1	87	87.3	83.7	87.7	100

1: CCA1-24 (HQ179141), 2: CWP1-6-1(HQ179147), 3: CSP2-25-1-1(HQ179142), 4: SC4 (HQ179137), 5: CASA51-1(HQ179148), 6: *Bacillus subtilis* 224 (GU230167), 7: *B. subtilis* ANctcri3 (HQ286641), 8: *B. subtilis* KQC 85 (HM585058), 9: *B. subtilis* PAE 11

(HQ678671), 10: *B.subtilis* TAT 2-8 (HQ236067), 11: *B. subtilis* PARZ 2 (HQ678655), 12: *B. cereus* ATCC 43881 ^{T} (AF290550), 13: *B. cereus* TA2 (GQ381280), 14: *B. cereus* IMAUB 1022 (FJ641009), 15: *B. cereus* DZ4 (HQ143564), 16: *B. cereus* MZQ-ZJ01(GQ407269), 17: *B. cereus* WYLW1-9 (HM003213), 18: *B. cereus* ATCC 21281^{T} (FJ501984T), 19: *Bacillus sp.* PBCC 10 ^{T} (HM775374), 20: *B. cereus* EIF59 (HM480354), 21: *B. cereus* GCH-1 (FJ611966), 22: *B. cereus* BQAR-01d (FJ217203), 23: *B. cereus* SRS-30 (GU056810), and 24: *B. cereus* JS-33 16S(GQ280103).

Table 21 Identification results of selected PHAs-producing bacterial isolates based on API identification system (bioMérieux, Inc., France) compared to 16S rRNA gene sequence.

Group	Bacterial isolate code	Cell shape/arrangement	Cell size (µm)	Identification (% identity)	
				API system (bioMérieux, Inc., France)	16S rRNA gene sequence (GenBank, U.S.A.)
1	PCA1-10-1	Rods, short chain	0.3-0.5×2.0-2.4	*Listeria welshimeri* (99.9%)	Uncultured bacterium clone IMAU 374 GQ267950 (99%)
2	CSP2-3	Rods, singly, short chain	0.2-0.5×1.2-1.3	*Chryseobacterium indologenes* (99.7%)	*Pseudomonas aeruginosa* PAON 2 EF051248 (96.7%)
	CSP2-21	Rods, singly, short chain	0.5-0.7×1.7-3.1	*C.indologenes* (99.8%)	*Klebsiella oxytoca* SB 9 AJ871855 (99.1%)
	CAS5-1	Rods, singly, short chain	0.4-0.5×0.7-1.1	*C. indologenes* (89.6%)	*Enterobacter hormaechei* CIP 103441 ^{T} AJ508302 (99.5%)
3	SC4	Rods, chain	0.8-1.1×2.1-2.5	*Bacillus cereus* (99.9%)	*Bacillus cereus* ATCC 43881 ^{T} AF290550 (99.2%)
	CASA51-1	Rods, chain	0.5-0.7×2.1-3.1	*B. firmus* (99%)	*Bacillus* sp. PBCC 10 ^{T} HM775374 (87.2%)
	CST2-2	Rods, chain	1.1-1.5×2.5-3.3	*B. megaterium* (99.4%)	*Bacillus* sp. IMT 21^{T} GU479395 (99.2%)
	CWC1-6-1	Rods, chain	0.9-1.1×2.5-3.1	*B. mycoides* (95.4%)	*B. cereus* ATCC 21281^{T} FJ501984 (99%)
	CSP2-25-1-1	Rods, chain	1.1-1.2×3.1-3.5	*B. stearothermophilus* (99.7%)	*B. cereus* ATCC 43881 ^{T} AF290550 (98%)
	CCA2-11	Rods, chain	1.2-1.5×2.4-3.6	*B. megaterium* (99.7%)	*B. megaterium* ATCC 14581^{T} GU252112 (99.9%)
	CSP2-25-1	Rods, chain	0.7-1.0×2.7-3.2	*B. cereus* (97.9%)	*Bacillus* sp. NCCP-158 ^{T} AB560659 (99.7%)
	CCA1-24	Rods, chain	0.8-1.2×2.0-2.5	ND	*B. subtilis* ANctcri3 HQ286641 (99.6%)
	CSC2-2	Rods, chain	1.1-1.2×3.1-3.3	*B. cereus* (96.8%)	ND
	CSP2-23	Rods, chain	0.9-1.3×3.1-4.0	*B. stearothermophilus* (98.8%)	ND
	CSP2-26-1	Rods, chain	0.4-0.7×1.1-1.4	*B. firmus* (93.1%)	ND
4	CWP2-16	Cocci, pair, cluster	0.4-0.5×0.4-0.5	*Staphylococcus cohnii* subsp. *urealyticum* (99.6%)	*Staphylococcus cohnii* JL 812 EF512729 (99.5%)
	CCS1-13	Cocci, pair, cluster	0.5-0.7×0.5-0.7	*S. lentus* (99.9%)	ND
	BCC2-7	Cocci, pair, cluster	0.5-0.8×0.5-0.8	*S. sciuri* (85.7%)	ND
	CCA1-24	Cocci, pair, cluster	0.5-0.7×0.5-0.7	*Micrococcus* sp.(99.7%)	ND
	PCA1-13	Cocci, pair, cluster	0.7-0.9×0.7-0.9	*S. xylosus* (94.9%)	ND

Table 22 Similarity of 16S rRNA gene sequence of selected PHAs-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details			
		Closest relative	Length of sequence (bp)	Sequence homology (%)	Isolation source / remark of closest relative
PCA1-10-1	1435	Uncultured bacterium clone IMAU 374 (GQ267950)	1473	99	Fermented milk
		Uncultured bacterium clone IMAU 336 (GQ267941)	1472	99	Fermented milk
		Uncultured bacterium clone IMAU 303 (GQ267933)	1472	99	Fermented milk
		Uncultured bacterium clone IMAU 250 (GQ267913)	1472	99	Fermented milk
CSP2-3	1406	*Pseudomonas aeruginosa* PAON 2 (EF051248)	1430	96.7	Data not show
		P. aeruginosa MTH 4 (HQ202540)	1393	96.7	Soil
		P. aeruginosa MTH 8 (HQ202541)	1393	96.5	Soil
		P. aeruginosa (EF530572)	1413	96.6	Data not show
		P. aeruginosa (X06684)	1537	95.6	Data not show
		P. aeruginosa KS-1 (GQ203616)	1491	96.5	Garden soil
CSP2-21	1407	*Klebsiella oxytoca* No.8 (AB353048)	1512	98.9	A fatal case of necrotizing
		K.oxytoca No.5 (AB353045)	1512	99.1	A fatal case of necrotizing
		K. oxytoca SB 9 (AJ871855)	1454	99.1	Fossil
		K.oxytoca (AY150697)	1434	98.9	Data not show
		K. oxytoca (AF129440)	1436	98.1	Data not show
CAS5-1	1439	*Enterobacter ludwigii* EN-119^{T} (AJ853891)	1513	98.6	Mid-stream urine
		E. cloacae ATCC 13047^{T} (AJ251469)	1511	98.3	Data not show
		E.hormaechei CIP 103441^{T} (AJ508302)	1368	99.5	Data not show
		E. cowanii CIP 107300^{T} (AJ508303)	1362	98.2	Data not show
		E. asburiae (AB004744)	1422	98.7	Gut
		Enterobacter cancerogenus LMG 2693 (Z96078)	1495	98.2	Data not show
CASA51-1	1,453	*Bacillus* sp. PBCC 10^{T} (HM775374)	1487	87.2	Curd oil
		B. cereus SRS-30 (GU056810)	1504	88.2	Plant root
		B. cereus JS-33 (GQ280103)	1436	89.8	Effluent and sludge from soda ash industry
CWP1-6-1	1,443	*B. cereus* ATCC 43881^{T} (AF290550)	1482	99.0	Soil
		B. cereus ATCC 21281^{T} (FJ501984)	1457	99.0	Soil
		Bacillus sp. PBCC 10^{T} (HM775374)	1487	99.9	Crude oil

Table 22 (Continued) Similarity of 16S rRNA gene sequence of selected PHAs-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details			
		Closest relative	Length of sequence (bp)	Sequence homology (%)	Isolation source / remark of closest relative
CSP2-25-1-1	1,410	*B. cereus* ATCC 43881 ^{T} (AF290550)	1482	98.0	Soil
		B. cereus ATCC 21281 ^{T} (FJ501984T)	1457	98.0	Soil
		Bacillus sp. PBCC 10 ^{T} (HM775374)	1487	98.0	Curd oil
CSP2-25-1	1,410	*B. aryabhattai* LS15 (GU563349)	1443	99.7	Rhizosphere soil
		B. flexus NY-1 (EU869200)	1428	99.7	Pesticide contaminated soil
		Bacillus sp. IMT 21 ^{T} (GU479395)	1483	99.7	Pesticide contaminated soil
		Bacillus sp. NCCP-158 ^{T} (AB560659)	1479	99.7	Diseased citrus leaves
CCA1-24	1,410	*B. subtilis* ANctcri3 (HQ286641)	1446	99.6	Rock surface
		B. subtilis KQC 85 (HM585058)	1419	99.6	Plant rhizosphere soil
		B. subtilis PAE 11 (HQ678671)	1437	99.6	Rhizosphere
		B.subtilis TAT 2-8 (HQ236067)	1414	99.6	Mineral and soil sample from mine
		B. subtilis PARZ 2 (HQ678655)	1434	99.6	Rhizosphere
CCA2-11	1,410	*B. megaterium* ATCC 14581 ^{T} (GU252112)	1495	99.9	Data not show
		B. megaterium LAMA 262 (HM104232)	1544	99.9	Residue of the processing of cassava
		B. megaterium 2008724133 (GU252120)	1495	99.9	Clinical host
		Bacillus megaterium PAB 1C5 (EU221343)	1531	99.9	Wheat rhizoshere
		Bacillus megaterium 2EJ4 (GQ383911)	1465	99.9	Rhizospere soil
		Bacillus megaterium PCWCW 5 (GQ284474)	1557	99.9	Water sample
		Bacillus megaterium MBFF 6 (HQ840732)	1502	99.9	Soil
CST2-2	1,410	*B. aryabhattai* F77063 (HQ908708)	1480	99.2	Sponges
		Bacillus sp. IMT 21 ^{T} (GU479395)	1483	99.2	Pesticide contaminated soil
		Bacillus sp. NCCP-158 ^{T} (AB560659)	1479	99.2	Diseased citrus leaves
		Bacillus sp. CCBAU 51492 ^{T} (EU621905)	1407	98.5	Ornamental plant grown in flowerpot
SC4	1,410	*B. cereus* ATCC 43881 ^{T} (AF290550)	1482	99.2	Soil
		B. cereus ATCC 21281 ^{T} (FJ501984T)	1475	99.2	Soil
		Bacillus sp. PBCC 10 ^{T} (HM775374)	1487	99.1	Crude oil

Table 22 (Continued) Similarity of 16S rRNA gene sequence of selected PHAs-producing bacterial isolates compared with other bacteria from nucleotide sequence database (NCBI).

Bacterial isolate code	Length of sequence (nt)	Nucleotide sequence comparison, identification result and details			
		Closest relative	Length of sequence (bp)	Sequence homology (%)	Isolation source / remark of closest relative
CWP2-16	1381	*S. cohnii* JL 812 (EF512729)	1412	99.5	Marine environment
		S. cohnii subsp. *urealyticus* CTDE 2 (GQ169065)	1428	99.5	Deep sea sediment
		S. cohnii 175 (EU730931)	1450	99.4	Water
		S.cohnii (AB009936)	1494	99.5	Data not show
		S. cohnii subsp. *urealyticus* CK 27 (NR_037046)	1494	99.5	Data not show
		S.. nepalensis CW 1^T (AJ517414)	1470	99.1	Host lung and nose
		S. cohnii subsp. *cohnii* ATCC 29974^T (D83361)	1477	99.3	Animal

Note: ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; PBCC, Petroleum Biotechnology Culture Collection; MIT, Micoteca do Institute de Medicina Tropical de Sao Paulo; NCCP, National for Culture Collection for Pathogens.

When combining results from morphological and physiological characteristics, and 16S rDNA sequence, 8 genera: *Bacillus*, *Chryseobacterium*, *Enterobacter*, *Klebsiella*, *Listeria* (non-pathogenic species), *Micrococcus*, *Pseudomonas*, and *Staphylococcus* were classified. Genera of PHAs-producing bacteria isolated from cassava pulp were similar to bacteria found in other habitats, such as *P. fluorescens*, and *P. aeruginosa* isolated from activated sludge (Ciesielski *et al*., 2006); *P. aeruginosa* isolated from saline water (Yurkova *et al*., 2002)*; P. stutzeri* isolated from oil-contaminated soil (He *et al*., 1998); *P. mendocina*, a proteobacterium from oil-contaminated soil samples (Zheng *et al*., 2005). Bacteria belonging to genera *Pseudomonas, Aeromonas, Bacillus*, *Enterobacter*, *Acinetobacter*, and *Exiguobacterium* were found in agricultural field soil (Full, Jung, and Madigan, 2006), and *Bacillus* sp. was found in gas field soil (Tajima *et al*., 2003).

4.5 PHA production in controlled fermenter

Isolate CCA2-11 was chosen to produce PHAs in a controlled fermenter (6.6 L with working volume of 5.0 L) using fed batch cultivation. The suitable complex medium (2.5 L) was used to provide growth of the bacteria, then 2.5 L of minimal medium were added. Growth of the strain CCA2-11 increased from 10^5 CFU/mL to 10^8 CFU/mL during 48 h cultivation (Table 4.23). The bacterial cells were harvested after feeding the suitable minimal medium at 48 h cultivation and further cultured till 96 h. PHAs were extracted from the cells, and the product yield of 0.254 g/5L of medium was obtained (Table 4.24).

Table 4.23 Growth of isolate CCA2-11 in fed-batch culture in 6.6 L fermenter at 96 h.

Cultivation time (h)	Culturing medium[a]	Growth rate (CFU/mL)
0	Modified complex medium	1.75×10^{5}
24	Modified complex medium	2.00×10^{8}
48	Modified complex medium	1.20×10^{8}
48	Modified complex medium	4.00×10^{6}
72	Modified minimal medium	2.50×10^{6}
96	Modified minimal medium	3.50×10^{6}

[a] At 48 h of cultivation, an equal volume of modified minimal medium was added to the complex medium.

Table 4.24 Extraction of PHAs from cells of isolate CCA2-11 using 1,2-Dichloroethane.

Product	Weight (g)	Product appearances
Wet cell	91.888	Dark brown, creamy, and odor
Cellular dry weight	8.695	Dark brown, soft, and puffy
PHAs	0.254	Sticky

It seems that this extraction method provided very low amount of PHAs when compared to PHA granules within their cells observed under fluorescence microscope (85.77%) and detected by TEM (Figure 4.7 D) PHA extraction and purification methods should be developed for the selected Gram-positive bacterium which had a thick cell wall.

CHAPTER V

CONCLUSION

Seven hundred and seventy two bacterial isolates obtained from the Microbial Culture Collection and Applications Research Unit, Institute of Science, Suranaree University of Technology, and new isolates from cassava pulp samples collected from modified cassava starch industrial factories, and sun drying fields for cassava pulp were used for the investigation of the diversity of PHAs-producing bacteria. The majority of bacteria had their colony morphology to be circular with entire edge, smooth surface, umbronate elevation, and 0.1-0.3 cm in diameter. Most of them (98%) were Gram-positive, rod shape and spore-forming bacteria with sizes of their cells ranging from 0.4-0.7×1.1-1.4 to 0.77-1.05×3.67-6.38 μm. These bacteria could be grouped into 4 groups: (1) regular, non-sporing Gram-positive rod bacteria, (2) Gram-negative rod bacteria, (3) endospore-forming Gram-positive rod bacteria, and (4) Gram-positive coccus bacteria. Appropriate media for the detection of PHA accumulation in the bacterial cells were investigated for each group, based on methods that have been reported. It was found that the suitable complex medium was composed of (per liter) 5 g of yeast extract, 5 g of polypeptone, 5 g of tryptone, 2.5 g of NaCl, and 10 g of glucose; and the minimal medium composed of 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of ferrous ammonium citrate, 10 g of glucose, 1 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3 g of Na_2HPO_4, 1 g of $(NH_4)_2SO_4$, 1 mL of trace element solution containing (0.2 g of $CoCl_2 \cdot 6H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 5.56 g of

$FeSO_4 \cdot 7H_2O$, 0.3 g of H_3BO_3, 0.03 g of $MnCl_2 \cdot 4H_2O$, 0.03 g of $NaMoO_4 \cdot 2H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$). Phosphate that has been reported to be influence the accumulation of PHAs was also investigated. The optimized minimal medium containing KH_2PO_4 and Na_2HPO_4 at 1 and 3 g/L was found to be suitable for the detection of PHAs-producing bacteria isolated from cassava pulp. The bacterial isolates were cultured on the suitable complex and minimal media for screening of PHA accumulation in their cells and incubation at 30°C for 48 h. Nile red dyeing technique was introduced for the detection of PHAs-producing bacteria. The dye 0.00005% dissolved in DMSO was added directly to the optimized minimal medium. *Alcaligenes eutrophus* TISTR 1095 was used as the positive control of PHAs-producing strain. *Escherichia coli* TISTR 527 was utilized for negative control. Pink colonies under UV light of bacteria grown on the medium were selected for detection of PHA in their cells by staining with 1% Nile blue A and observed under fluorescence microscope at wavelength of 650 nm. Eighty two isolates were found to accumulate PHAs approximately between 3.3 to 85.77% within their cells. PHAs-producing bacteria isolated from cassava pulp were monitored of PHA accumulation in their cells using Nile red dyeing technique during cultivation. The strains could produce PHA of a highly yield at 48-72 h and PHA was decreased at 96 h. PHA granules were also observed under TEM and SEM. For the electron microscopy technique, the specimen preparation method was improved. Fixative solution and dehydration series were especially developed for PHA which was lipid compound. The fixative solution composing of 5% glutaraldehyde, 1% OsO_4 and 0.2 M phosphate buffer was found to be suitable. Acetone at series (20, 40, 60, 80, and 100%) gave better results than ethanol in the dehydration step. The diversity of PHAs-

producing bacteria isolated from cassava pulp was obtained from identification and characterization of the PHAs-accumulating bacteria. PHAs-producing bacteria were identified by morphological and physiological characteristics, and 16S rRNA gene sequence. From cell morphology, the bacteria could be divided into 4 groups, which were regular, non-sporing Gram-positive rod, Gram-negative rod, endospore-forming Gram-positive rod, and Gram-positive coccus groups. When combined results from morphological, physiological, and 16S ribosomal rRNA gene sequence characterization, 8 genera: *Bacillus*, *Chryseobacterium*, *Enterobacter*, *Klebsiella*, *Listeria* (non-pathogenic species), *Micrococcus*, *Pseudomonas*, and *Staphylococcus* were classified. These bacteria could have potential for further application in biopolymer production. Data achieved from this study are useful for the application of these bacteria.

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENT PREPARATION

1. Culture media for PHAs-producing bacteria

All components of each medium were added to distilled water mixed thoroughly and gently heated until dissolved, and brought volume up to 1.0 L. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

1.1 Developed complex medium

Yeast extract	5.0	g
Polypeptone	5.0	g
Tryptone	5.0	g
NaCl	2.5	g
Glucose	10.0	g
Agar	15.0	g

pH = 7.0 at 25°C

1.2 Developed minimal medium and trace element solution

$CaCl_2 \cdot 2H_2O$	0.01	g
Ferrous ammonium citrate	0.05	g
Glucose	10.0	g
KH_2PO_4	1.0	g

$MgSO_4 \cdot 7H_2O$	0.2	g
Na_2HPO_4	3.0	g
$(NH_4)_2SO_4$	1.0	g
Agar	15.0	g

pH = 7.0 at 25°C

Trace element solution per letter

$CoCl_2 \cdot 6H_2O$	0.2	g
$CuSO_4 \cdot 5H_2O$	0.01	g
$FeSO_4 \cdot 7H_2O$	5.56	g
H_3BO_3	0.3	g
$MnCl_2 \cdot 4H_2O$	0.03	g
$NaMoO_4 \cdot 2H_2O$	0.03	g
$NiCl_2 \cdot 6H_2O$	0.02	g
$ZnSO_4 \cdot 7H_2O$	0.1	g

The reagent was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation and kept at 4°C.

1.3 Carboxymethylcellulose (CMC) agar

Cellulose, ball milled	1.0	g
KH_2PO_4	1.0	g
$(NH_4)_2SO_4$	0.5	g
L-Asparagine	0.5	g
KCl	0.5	g

Yeast extract	0.1	g
$MgSO_4$	0.2	g
$CaCl_2$	0.1	g
Agar	15.0	g

pH = 6.2 ± 0.2 at 25°C

1.4 Thermo carboxymethylcellulose (TCMC) agar

KH_2PO_4	1.65	g
NH_4SO_4	1.6	g
Yeast extract	1.0	g
NaCl	0.96	g
L-Cysteine $HCl \cdot H_2O$	0.5	g
$MgSO_4$	0.096	g
$CaCl_2$	0.096	g
Cellulose suspension	200.0	mL
Resazurin (0.1% solution)	1.0	mL
Agar	15.0	g

pH = 7.2 ± 0.2 at 25°C

Preparation of cellulose suspension, cellulose powder was added 8.0 g to 200.0 mL of distilled water, mixed thoroughly, and filtrated through filter paper.

1.5 Plate count agar (PCA)

Casein enzymic hydrolysate	5.0	g
Yeast extract	2.5	g
Dextrose	1.0	g
Agar	15.0	g

pH = 7.0 ± 0.2 at 25°C

1.6 Trypticase soy agar (TSA)

Trypticase or Tryptose (Pancreatic digest of casein)	17.0	g
Phytone (Papaic digest of soya meal)	3.0	g
NaCl	5.0	g
KH_2PO_4	2.5	g
Glucose	2.5	g
Agar	15.0	g

pH = 7.3 ± 0.2 at 25°C

1.7 Starch agar (SA)

Nutrient agar	23.0	g
Starch	10.0	g
Distilled water	1.0	L

Preparation of starch suspension was added starch powder 10.0 g to 250.0 mL of distilled water. Nutrient agar and 3.0 g of agar were dissolved in 500.0 mL distilled water.

1.8 Trypticase soy broth (TSB)

Trypticase or Tryptose (Pancreatic digest of casein)	17.0	g
Phytone (Papaic digest of soya meal)	3.0	g
NaCl	5.0	g
KH_2PO_4	2.5	g
Glucose	2.5	g

pH = 7.3 ± 0.2 at 25°C

1.9 Triple sugar iron (TSI)

Peptic digest of animal tissue	10.0	g
Casein enzyme hydrolysate	10.0	g
Yeast extract	3.0	g
Beef extract	3.0	g
Lactose	10.0	g
Sucrose	10.0	g
Dextrose	1.0	g
NaCl	5.0	g
Ferric sulfate	0.2	g
Sodium thiosulfate	0.3	g
Phenol red	0.024	g
Agar	12.0	g

pH = 7.4 ± 0.2 at 25°C

All ingredients except carbohydrates were added to distilled water and gently

heated until dissolved. Then, the media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches. The medium was allowed to cool down to 45-50°C and added the sterile carbohydrates into medium.

1.10 Lysine indole motile (LIM) medium

Peptone	10.0	g
Tryptone	10.0	g
Yeast extract	3.0	g
L-lysine hydrochloride	10.0	g
Dextrose	1.0	g
Ferric ammonium citrate	0.5	g
Bromcresol purple	0.02	g
Agar	2.0	g

pH = 6.6 ± 0.2 at 25°C

1.11 Methyl Red Voges-Proskauer (MR-VP) medium

Peptone	5.0	g
Glucose	5.0	g
K_2PO_4	5.0	g

pH = 7.5 ± 0.2 at 25°C

1.12 Simmons citrate agar

$MgSO_4$	0.2	g
$(NH4)_2PO_4$	1.0	g
K_2PO_4	1.0	g
Sodium citrate	2.0	g
NaCl	5.0	g
Agar	15.0	g
Bromthymol blue	0.05	g

pH = 6.8 ± 0.2 at 25°C

1.13 Nitrate reduction broth

Peptone	5.0	g
Beef extract	3.0	g
KNO_3	1.0	g

pH 6.9 ± 0.2 at 25°C.

1.14 Oxidation-Fermentation medium

NaCl	5.0	g
Pancreatic digest of casein	2.0	g
K_2HPO_4	0.3	g
Bromthymol Blue	0.03	g
Agar	2.5	mL
Glucose solution	100.0	mL

Preparation of glucose solution: glucose powder (10.0 g) was added into 100.0 mL of distilled water. The solution was sterile by filtration. Medium preparation: all gradients were added into 900.0 mL of distilled water. The medium was mixed thoroughly and gently heated until dissolved, then sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches. The medium was allowed to cool down to 45-50°C and added with 100.0 mL of glucose solution.

1.15 Lauryl sulfate tryptose (LST) broth

Tryptose	20.0	g
Lactose	5.0	g
NaCl	5.0	g
Lauryl sulfate sodium salt	0.1	g
K_2HPO_4	2.75	g
KH_2PO_4	2.75	g

pH 6.8 ± 0.2 at 25°C

1.16 Motility test medium

Tryptone	10.0	g
Agar	5.0	g
NaCl	5.0	g

pH 7.2 ± 0.2 at 25°C

1.17 Nutrient gelatin

Peptone	5.0	g
Glucose	5.0	g
K_2PO_4	5.0	g
Gelatin	120.0	g

pH 7.5 ± 0.2 at 25°C

1.18 Phenol red broth

Proteose peptone	10.0	g
Phenol red	0.018	g

pH 7.4 ± 0.2 at 25°C

Proteose peptone was added in distilled water, then phenol red was added. The medium was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

1.19 Starch hydrolysis agar

Soluble starch	20.0	g
Beef extract	3.0	g
Tryptose	10.0	g
Agar	15.0	g

pH 7.4 ± 0.2 at 25°C

1.20 Tributyrin agar

Peptone	5.0	g
Beef extract	3.0	g
Tributyrin	10.0	g
Agar	15.0	g

pH 7.2 ± 0.2 at 25°C

1.21 Skim milk agar

Skim milk powder	100.0	g
Peptone	5.0	g
Agar	15.0	g

1.22 Lubia-Bertani (LB) broth

Tryptone	10.0	g
NaCl	5.0	g
Yeast extract	10.0	g

2. Chemicals and reagents for microbiological analysis

The reagents for conventional method of bacterial characterization were prepared as described by Cappuccino and Sherman (1999).

2.1 1% dimethyl-p-phenylenediamine dihydrochloride

Dimethyl-p-phenylenediamine dihydrochloride	1.0	g

Dimethyl-p-phenylenediamine dihydrochloride was dissolved in 100.0 mL. This reagent was kept in the refrigerator.

2.2 Gram staining reagents

2.2.1 Ammonium oxalate crystal violet

Crystal violet	2.0	g
95% Ethanol	20.0	mL
1% ammonium oxalate	80.0	mL

Crystal violet (2.0 g) was dissolved in 20.0 mL of ethanol (95%). Then, the mixture was transferred to 80.0 mL of 1% ammonium oxalate solution and mixed throughly.

2.2.2 Safranin O

Safranin O	0.25	g
95% Ethanol	10.0	mL
Distilled water	90.0	mL

Safranin O (0.25 g) was dissolved in 10.0 mL of 95% ethanol. Then, the mixture was diluted with 90.0 mL of distilled water

2.2.3 Iodine solution

Iodine	2.0	g
Potassium iodide	4.0	g
Distilled water	600.0	mL

Iodine (2.0 g) and potassium iodide (4.0 g) were dissolved in distilled water and made up volume to 600.0 mL. This solution was kept in the dark place.

2.2.4 95% Ethyl alcohol

Absolute ethanol	95.0	mL
Distilled water	5.0	mL

Absolute ethanol was added to distilled water, and mixed thoroughly.

2.3 3% hydrogen peroxide diluted

40% H_2O_2	7.5	mL
Distilled water	100.0	mL

The 40% H_2O_2 7.5 mL was diluted with distilled water to 100.0 mL.

2.4 Kovacs' reagent

p-Dimethylaminobenzaldehyde	5.0	g
Amyl alcohol	75.0	mL
Concentrated hydrochloric acid	25.0	mL

p-Dimethylaminobenzaldehyde (5.0 g) was dissolved in 75.0 mL of amyl alcohol at 50°C. The mixture was added to concentrated hydrochloric acid (25.0 mL). This reagent was kept in the dark bottle and stored in a refrigerator.

2.5 Methyl red solution

Methyl red	0.1	g
95% Ethanol	300.0	mL

Methyl red (0.1 g) was dissolved in 300.0 mL of 95% ethanol, and the total volume to 500.0 mL with distilled water.

2.6 Nitrate test reagents

Sulfanilic acid	0.8	g
5 N acetic acid	2.0	L
Dimethyl-α-napthylamine	5.0	g

Nitrate test reagents composed of solution A and B. For the preparation of solution A, sulfanilic acid (0.8 g) was dissolved in 1.0 L of 5 N acetic acid (1 part of glacial acetic acid in 2.5 parts of distilled water). For preparation of solution B, dimethyl-α-napthylamine (5.0 g) was dissolved in 1.0 L of 5 N acetic acid.

2.7 Voges-Proskauer test solution

Alpha-napthol	10.0	g
95% Ethanol	100.0	mL
KOH	20.0	g
Distilled water	100.0	mL

This solution was composed of two solutions: solution A and B. Solution A was prepared by diluting 10.0 g of alpha-napthol in 100.0 mL of 95% ethanol. Solution B was prepared by diluting 20.0 g of KOH in 100.0 mL of distilled water.

2.8 1% Nile blue A

Nile blue A	1.0	g
Distilled water	100.0	g

One gram of Nile blue A was dissolved in 100.0 mL distilled water. The solution was mixed thoroughly and filtrated passing filter paper and kept in the dark bottle.

2.9 Nile red

Nile red	0.5	mg
Dimethylsulfoxide (DMSO)	1.0	mL

Preparation of Nile red for medium agar, weighing of 0.5 mg of Nile red added in 1.0 mL of DMSO in test tube and poured into 1.0 L of modified minimal medium. The medium was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

2.10 Phosphate buffer (pH 7.2)

Na_2HPO_4, anhydrous	0.724	g
KH_2PO_4	0.210	g
Distilled water	1.0	L

Dissolve ingredients in distilled water. Adjust pH to 7.2 (with 1 N NaOH). The buffer was sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches

3. Chemical preparation for API identification system (bioMérieux, Inc., France)

3.1 McFarland turbidity standards

1% solution of anhydrous $BaCl_2$ and a 1% solution of H_2SO_4 were prepared and mixed together in various proportions as detailed in Table 1A to form a turbid suspension.

Table 1A Preparation of McFarland turbidity standards.

McFarland scale number	Amount of 1% $BaCl_2$ (mL)	Amount of 1% H_2SO_4 (mL)	Corresponding approximate density of bacteria (million/mL)
1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1,200
5	0.5	9.5	1,500
6	0.6	9.4	1,800
7	0.7	9.3	2,100
8	0.8	9.2	2,400
9	0.9	9.1	2,700
10	1.0	9.0	3,000

Source: Cappuccino and Sherman, (1999).

3.2 Sterile emulsion oil

Emulsion oil or parafilm oil 10.0 g

The oil solution was poured in glass vials with screw cap, and sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches before use.

4. Reagents for 16S rRNA gene

4.1 STE buffer

NaCl	2.92	g
Tris base	1.21	g
EDTA·$2H_2O$-Na_2	1.86	g

The final concentration of solution per litter was 100 mM NaCl, 10 mM Tris/HCl, and 1 mM EDTA. The solution pH was adjusted to 8.0 with NaOH, then autoclaved at 121°C, 15 lb/square inches for 15 min.

4.2 TE buffer

Tris Base	1.21	g
EDTA ($C_{10}H_{14}N_2O_8Na_2·2H_2O$)	0.37	g

The ingredients were dissolved. The volume was adjusted to 1.0 L with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches.

4.3 Tris-saturated phenol (pH 8.0)

Dissolved phenol was transferred to the mixture of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0

4.4 Loading buffer

Bromophenol blue	25.0	g

The dye was dissolved and adjusted the volume to 10.0 mL with 40% sucrose in water.

4.5 Ethidium bromide (10 mg/mL)

Ethidium bromide	1.0	g

The chemical was dissolved in sterilized deionized water and the volume was adjusted to 100.0 mL.

5. Chemicals and reagents for SEM and TEM analysis

5.1 Developed fixative solution

5.1.1 1% OsO_4

OsO_4	1.0	g
Distilled water	25.0	mL

One gram of OsO_4 was added into 25.0 mL of distilled water in the dark bottle and seal with parafilm, and then kept overnight in the dark place. OsO_4 solution (4%) was filtrated by filter paper. For preparation of 1% OsO_4, pipette one part of 4% OsO_4 was mixed with 3 parts of phosphate buffer. The solution kept in double dark bottle in flume hood.

5.1.2 5% Glutaraldehyde

50% Glutaraldehyde	10.0	mL

Pipette 10.0 mL of 50% glutaraldehyde into 100.0 mL volume metric flask and adjusted the volume using distilled water.

5.1.3 0.2M Phosphate buffer

Preparation of stock solution A and B for 0.2 M phosphate buffer

Solution A

$NaH_2PO_4 \cdot H_2O$ 27.60 g

Weighing of $NaH_2PO_4 \cdot H_2O$ 27.60 g added in distilled water and adjusted the volume to 1.0 L kept in dark bottle.

Solution B

Na_2HPO_4 28.40 g

Weighing of Na_2HPO_4 28.40 g added in distilled water and adjusted the volume to 1.0 L kept in dark bottle.

Table 2A Working solution of 0.2 M phosphate buffers.

Solution A (mL)	Solution B (mL)	pH
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5

5.2 4% Uranyl acetate (W/V)

Uranyl acetate	4.0	g
Distilled water or 70% ethanol	100.0	mL

Uranyl acetate (4.0 g) was dissolved in distilled water or 70% ethanol, kept overnight, then, filtrated and transferred to dark bottle kept in refrigerator.

5.3 Series of dehydration reagent

Table 3A Preparation of acetone series using for specimen dehydration.

Acetone concentration (%)	100% Acetone (mL)	Distilled water (mL)
20	20.0	80.0
40	40.0	60.0
60	60.0	40.0
80	80.0	20.0

5.4 Epon resin

Chemicals were weighed and mixed in plastic ware. The resin was sealed with parafilm and kept in refrigerator.

Epon-812 substitute	20.0	g
Dodecenyl succinic anhydride	20.0	g
Methyl-5-norbornene-2-3-dicarboxilic anhydride	6.0	g
2,4,6 (Tri(Dimethylaminoethyl)phenol)	0.7	g

5.5 0.3% Formvar

Formvar	0.3	g
Ethylene dichloride	100.0	mL

Formvar was added into 100.0 mL of ethylene dichloride, and kept in Erlenmeyer flask with screw cap kept in refrigerator.

5.6 Lead citrate (0.4%)

Lead nitrate	1.33	g
Sodium citrate	1.76	g
CO_2 free of distilled water	30.0	mL
1 N NaOH	8.0	mL

Lead nitrate and sodium citrate were mixed together for 30 min. NaOH (1 N) was added to the clear solution. The volume was then adjusted to 50.0 mL.

APPENDIX B

NUCLEOTIDE SEQUENCE DATA

1. Selected bacterial 16S rDNA sequences

Nucleotide sequence results of 16S rDNA were presented in dendrograms and nitrogen bases for example in Figures 1B to 24B using fD1 and rP2 primers.

1.1 Bacterial isolate CWP2-16

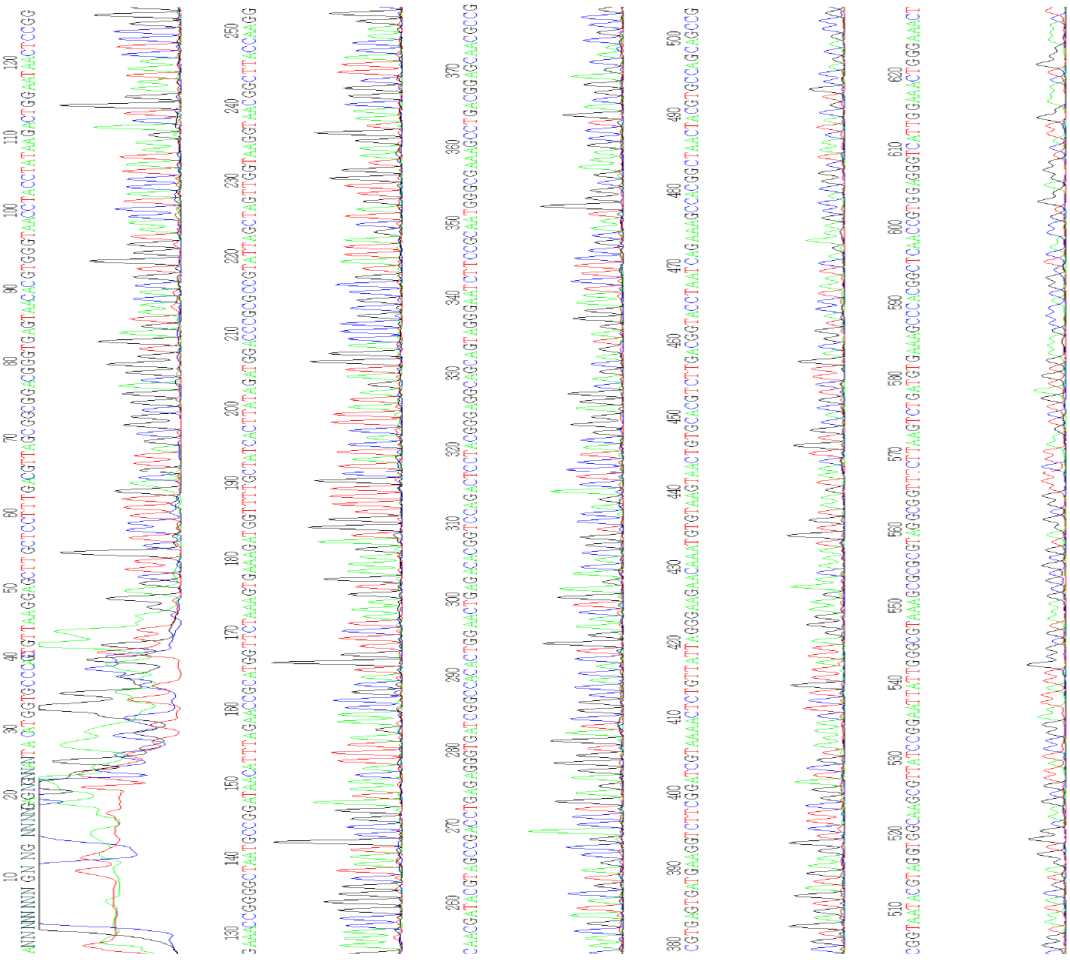

Figure 1B Sequence electrophenogram of 16S rDNA region of isolate CWP2-16 using fD1and rP2 primers.

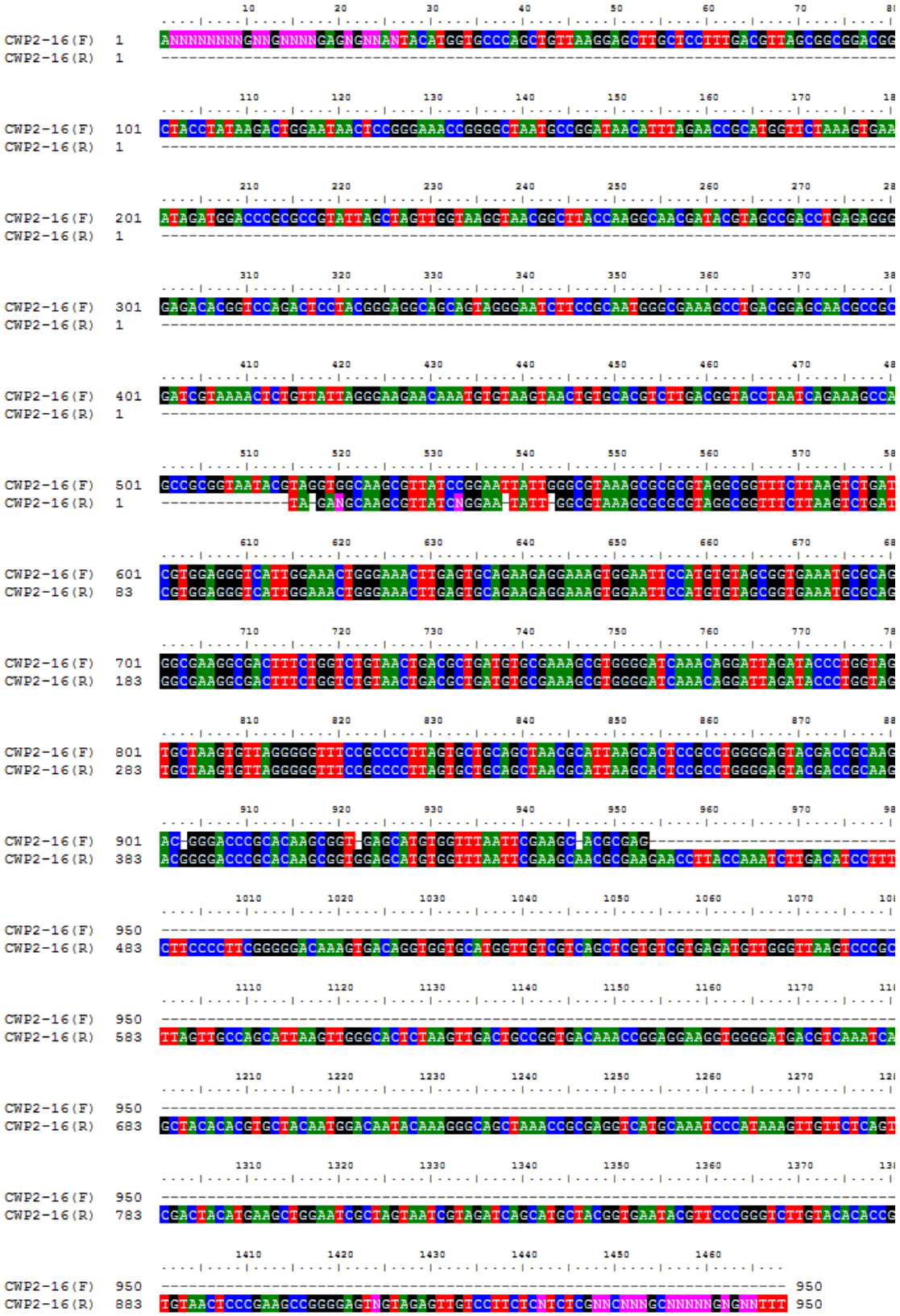

Figure 2B Sequence of 16S rDNA region of isolate CWP2-16 using fD1 and rP2 primers.

1.2 Bacterial isolate CSP2-3

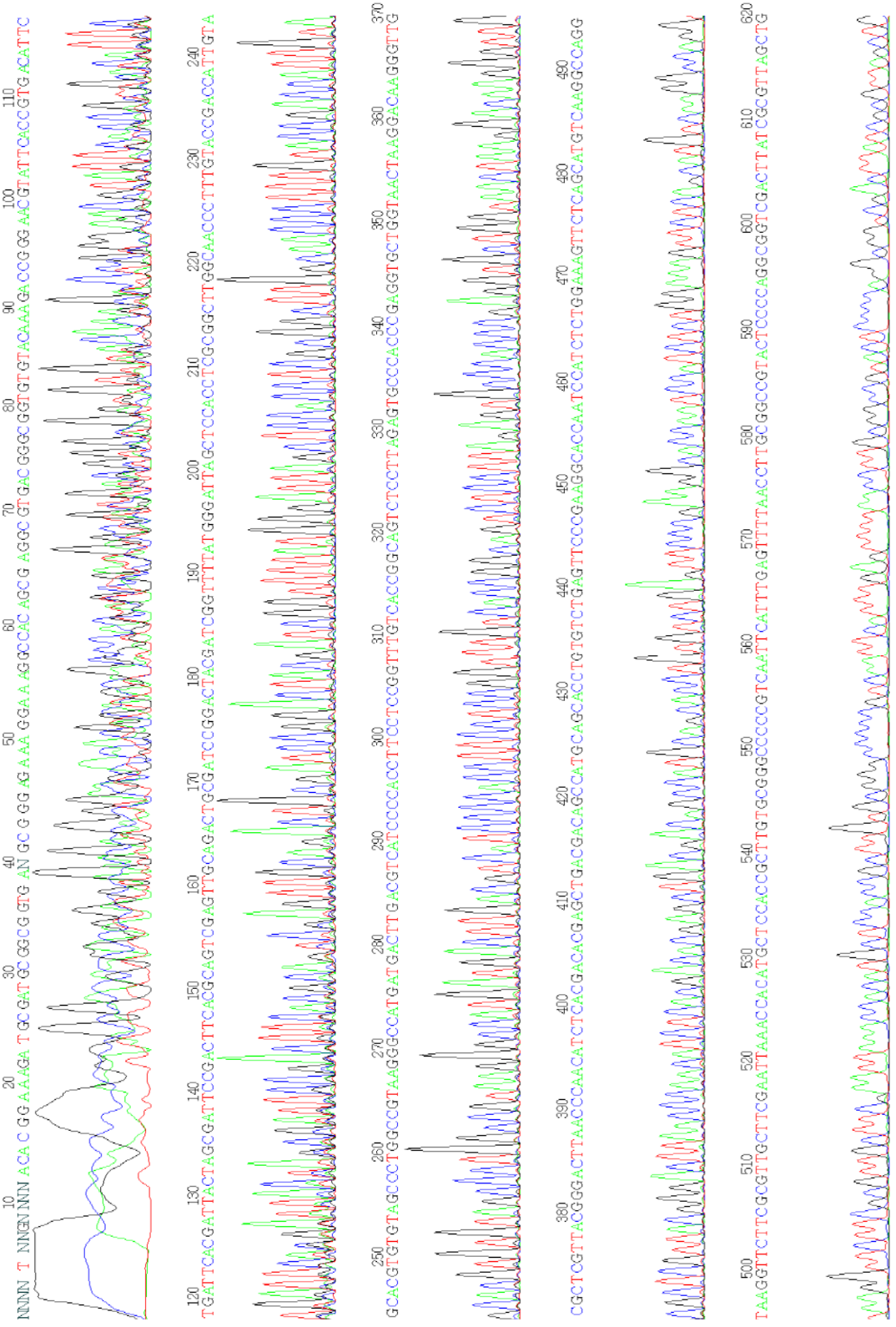

Figure 3B Sequence electrophenogram of 16S rDNA region of isolate CSP2-3 using fD1 and rP2 primers.

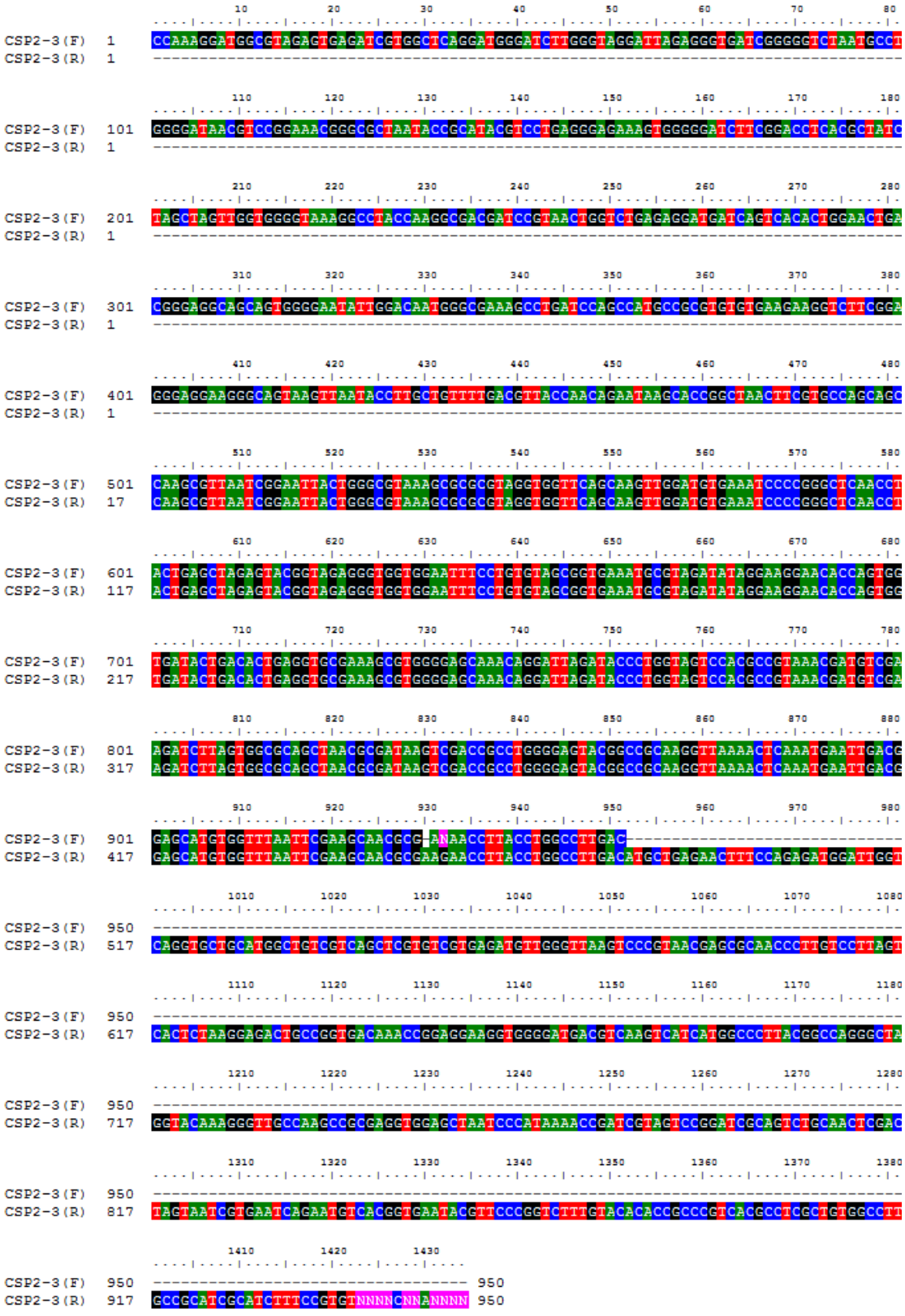

Figure 4B Sequence of 16S rDNA region of isolate CSP2-3 using fD1 and rP2 primers.

1.3 Bacterial isolate CAS5-1

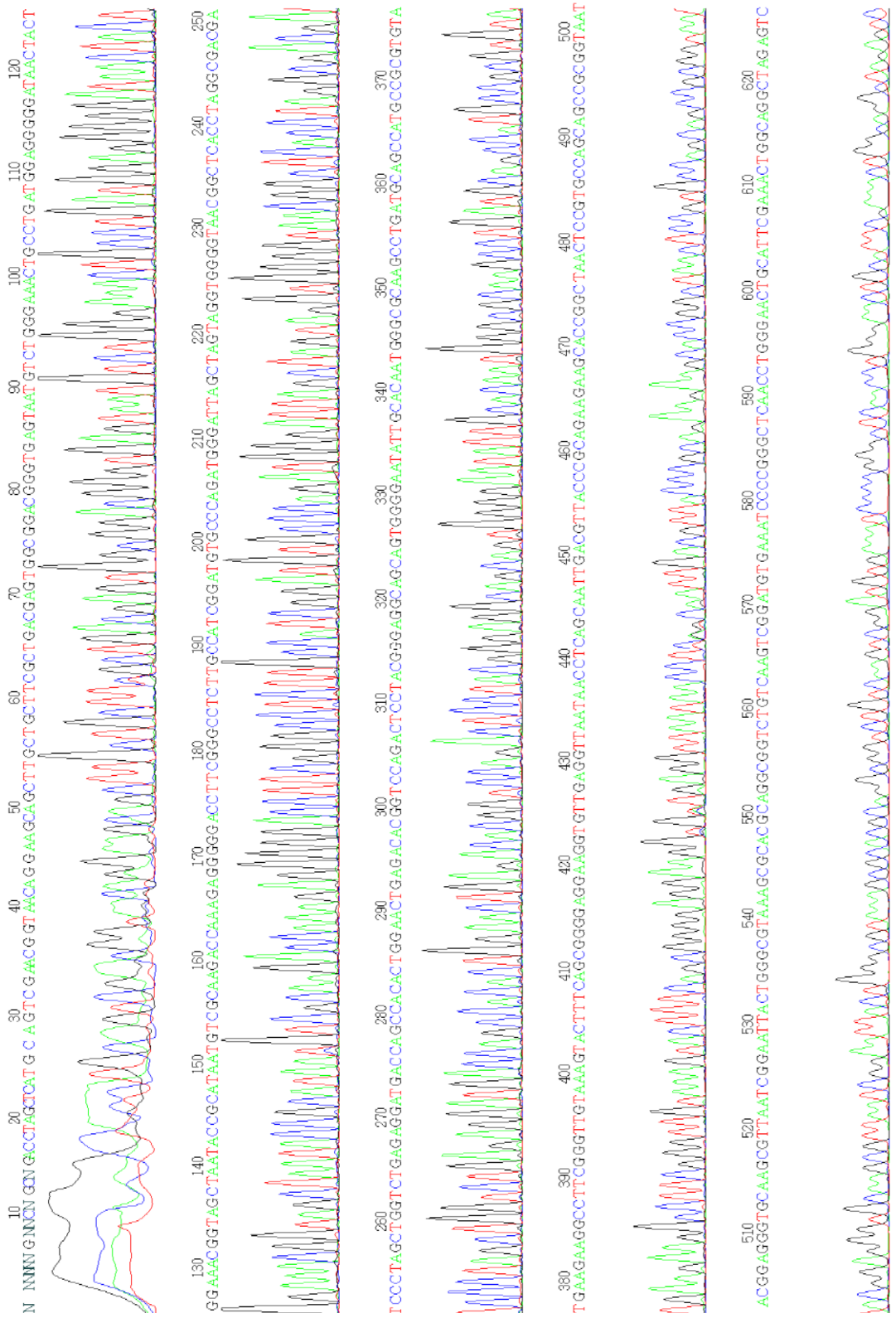

Figure 5B Sequence electrophenogram of 16S rDNA region of isolate CAS5-1 using fD1 and rP2 primers.

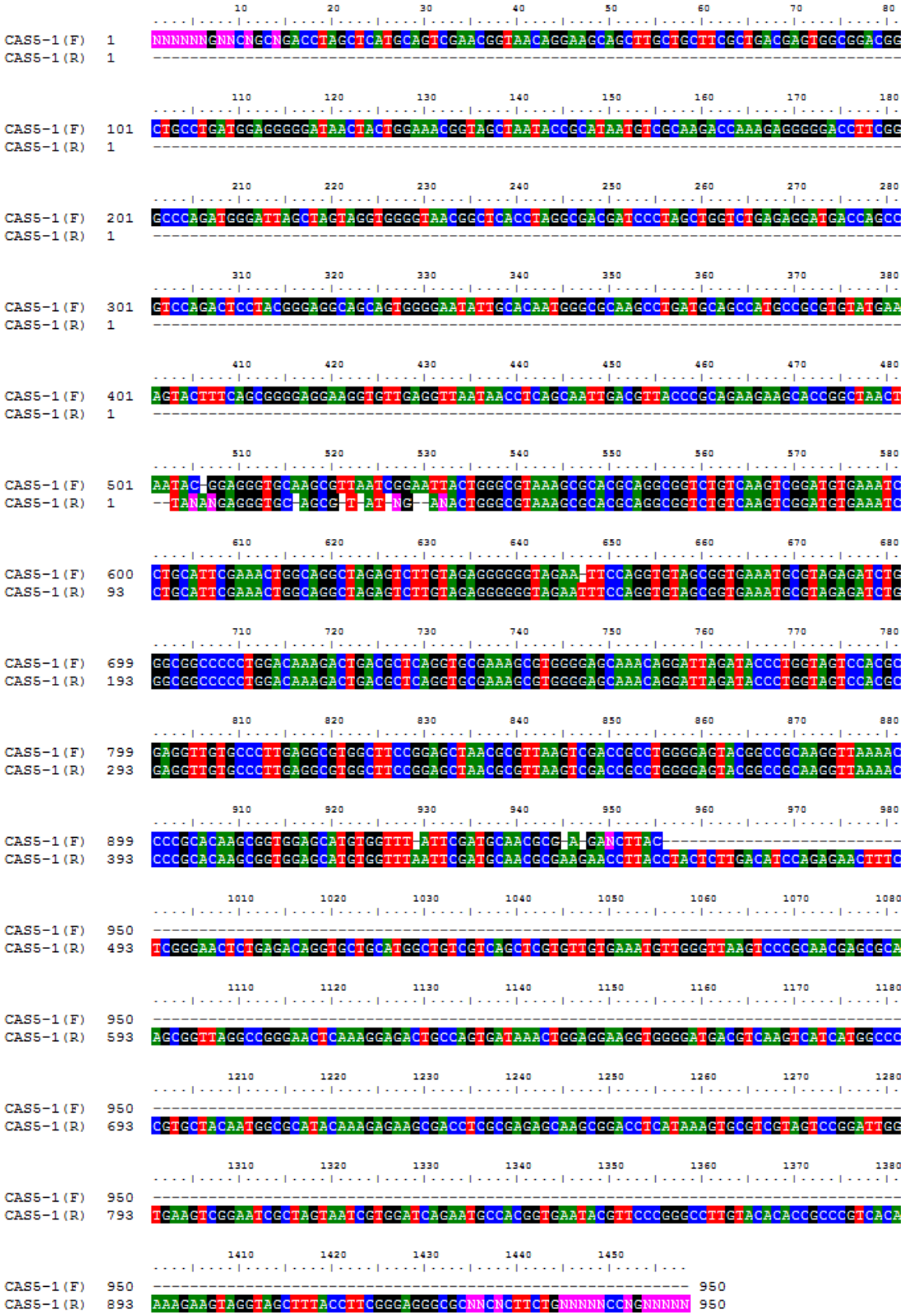

Figure 6B Sequence of 16S rDNA region of isolate CAS5-1 using fD1 and rP2 primers.

1.4 Bacterial isolate CSP2-21

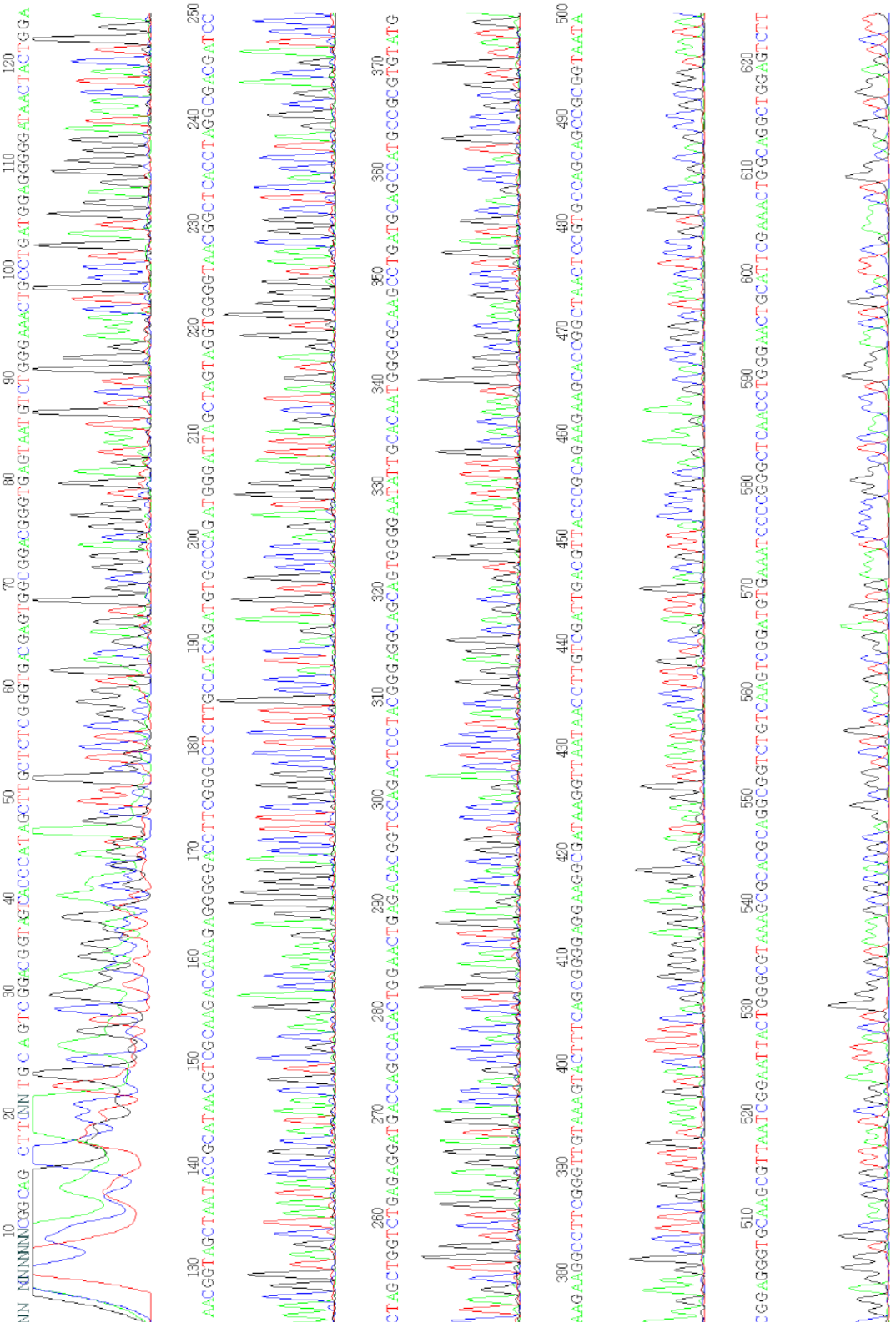

Figure 7B Sequence electrophenogram of 16S rDNA region of isolate CSP2-21 using fD1 and rP2 primers.

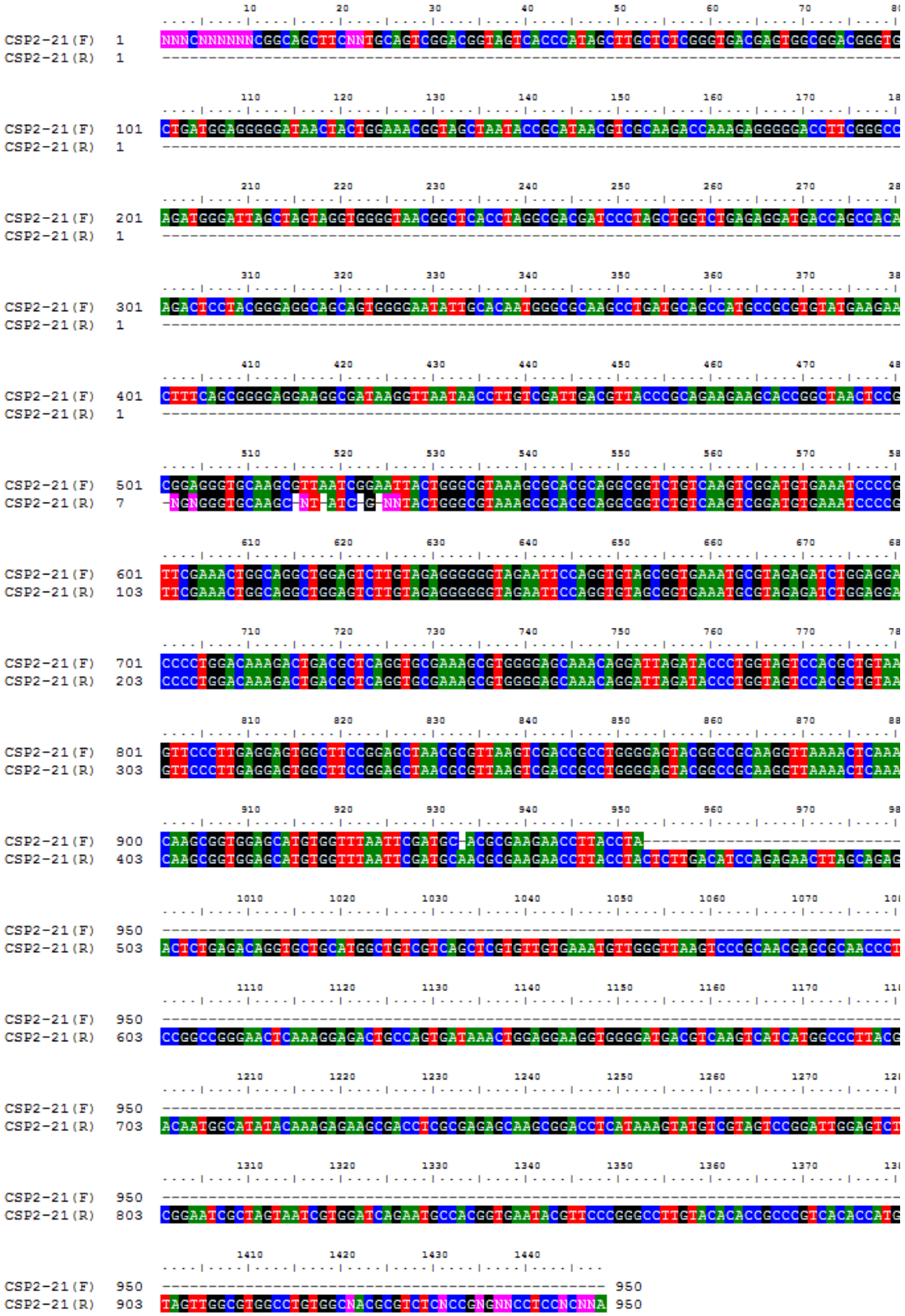

Figure 8B Sequence of 16S rDNA region of isolate CSP2-21 using fD1 and rP2 primers.

1.5 Bacterial isolate CASA51-1

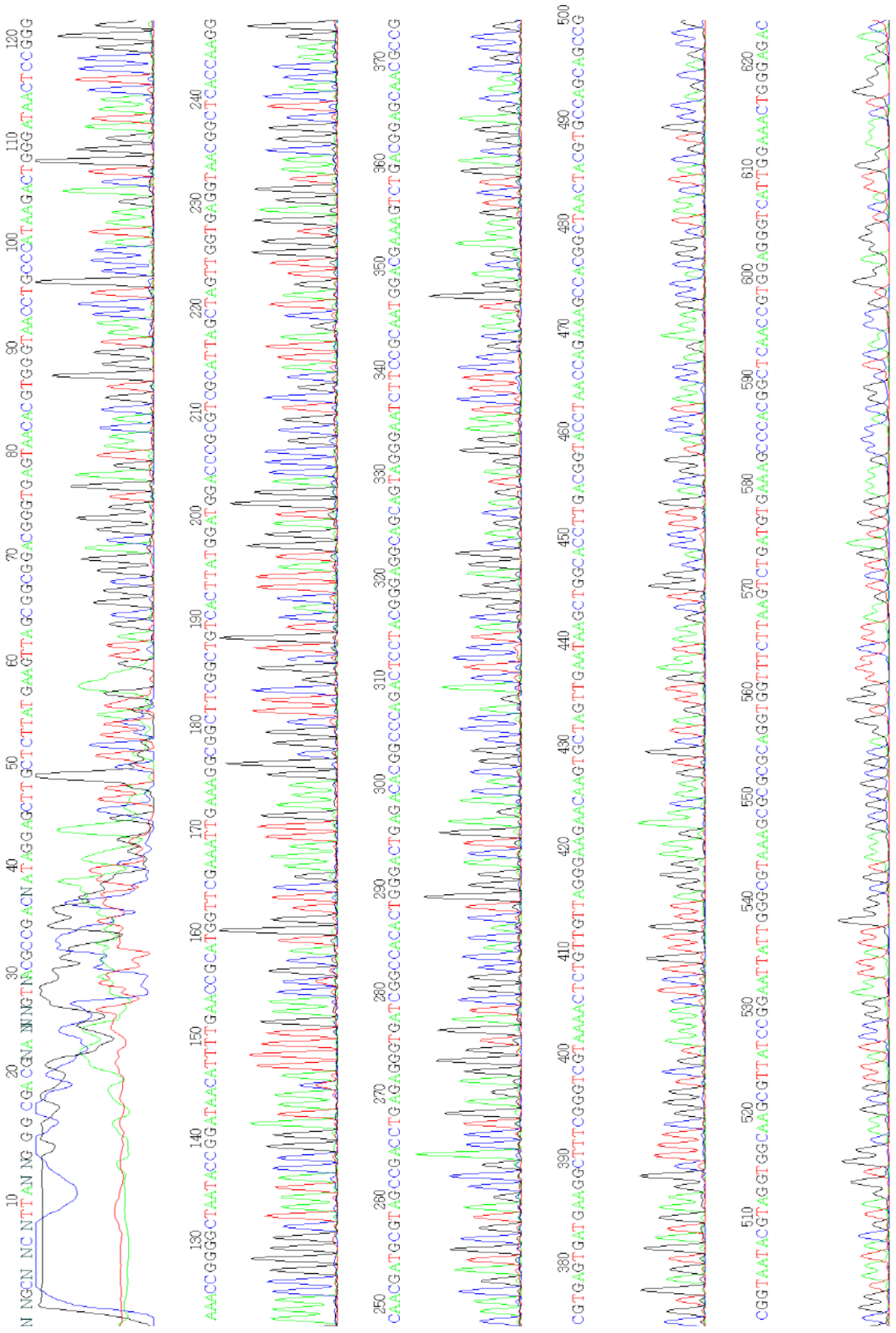

Figure 9B Sequence electrophenogram of 16S rDNA region of isolate CASA51-1 using fD1 and rP2 primers.

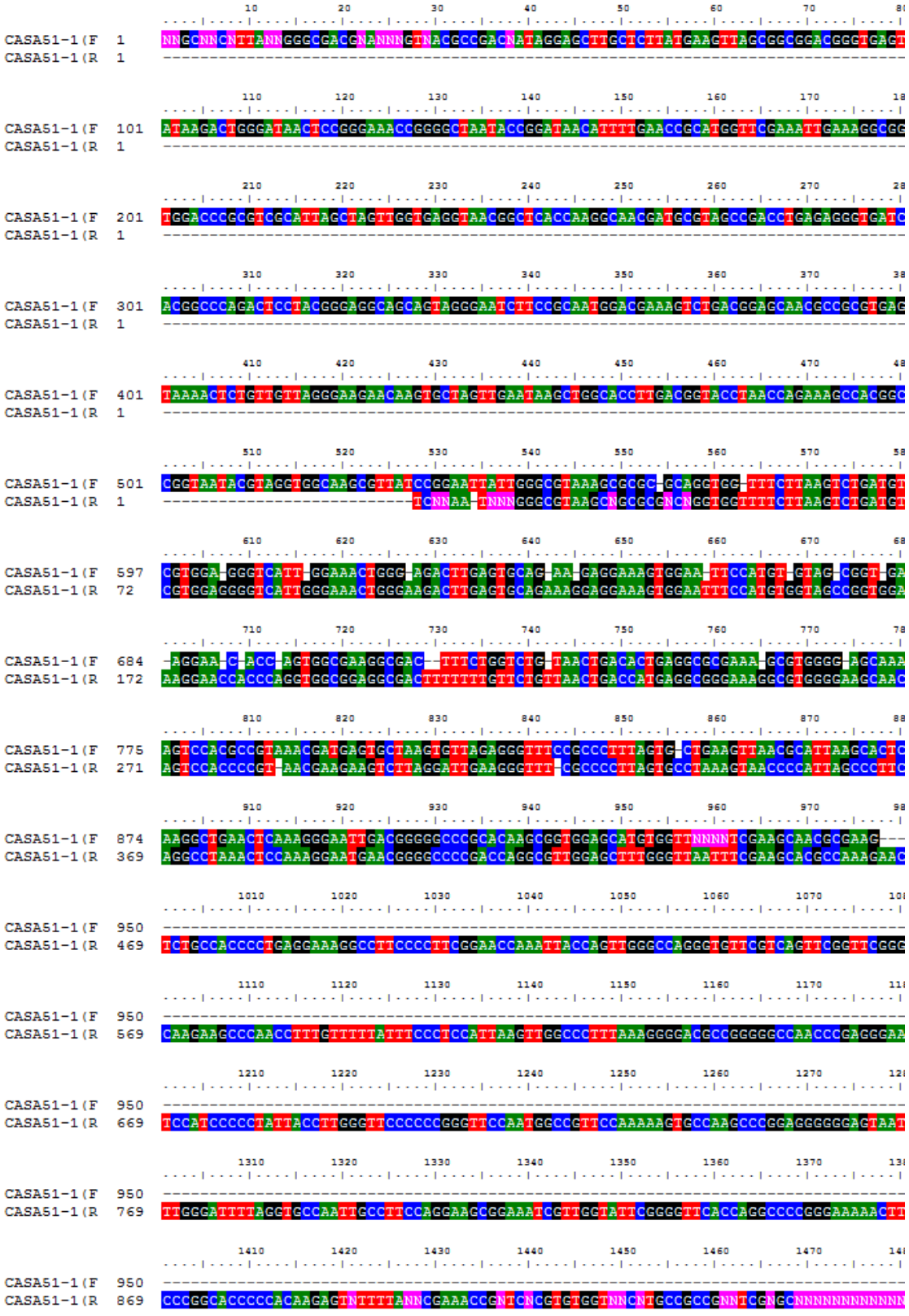

Figure 10B Sequence of 16S rDNA region of isolate CASA51-1 using fD1 and rP2 primers.

1.6 Bacterial isolate SC4

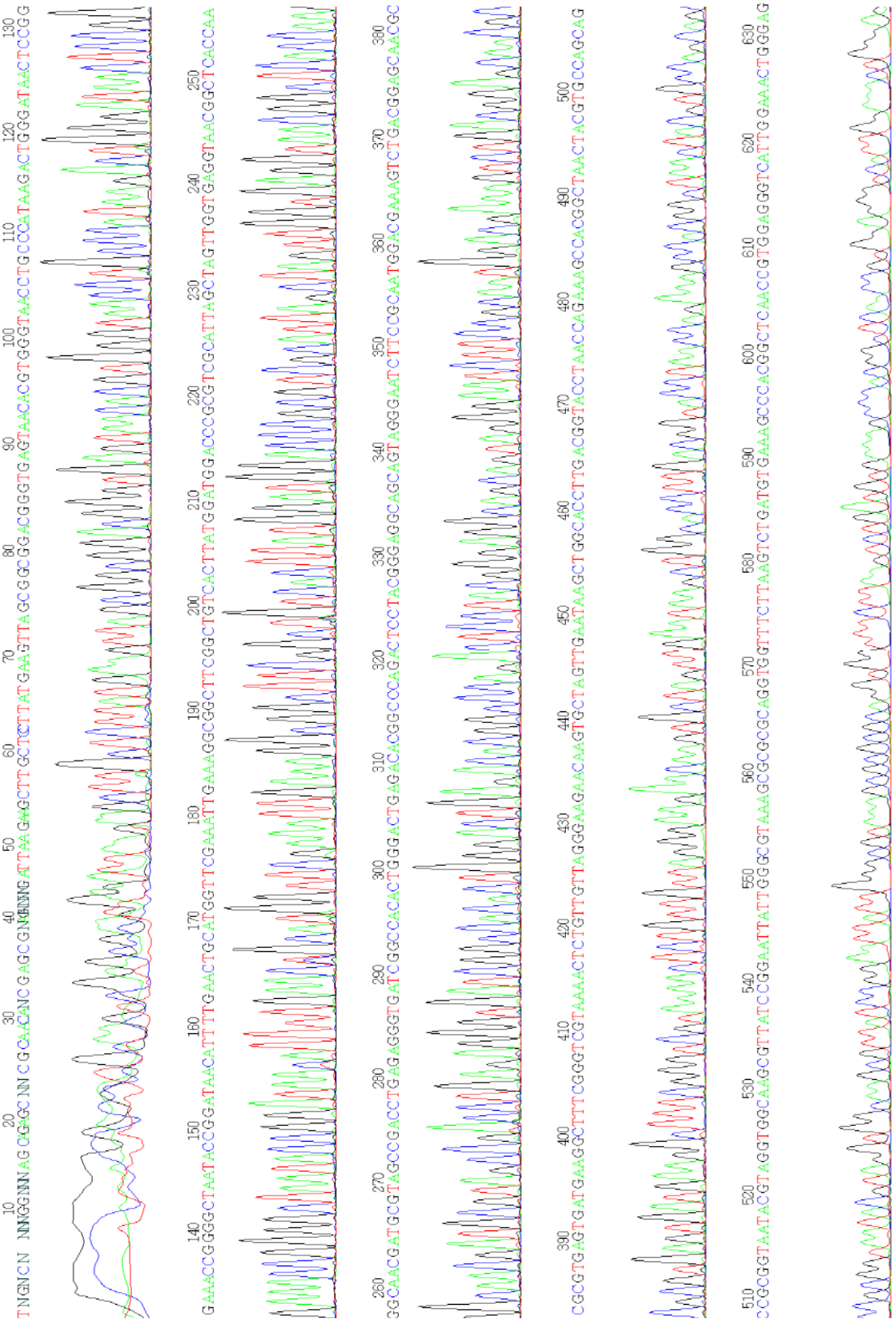

Figure 11B Sequence electrophenogram of 16S rDNA region of isolate SC4 using fD1 and rP2 primers.

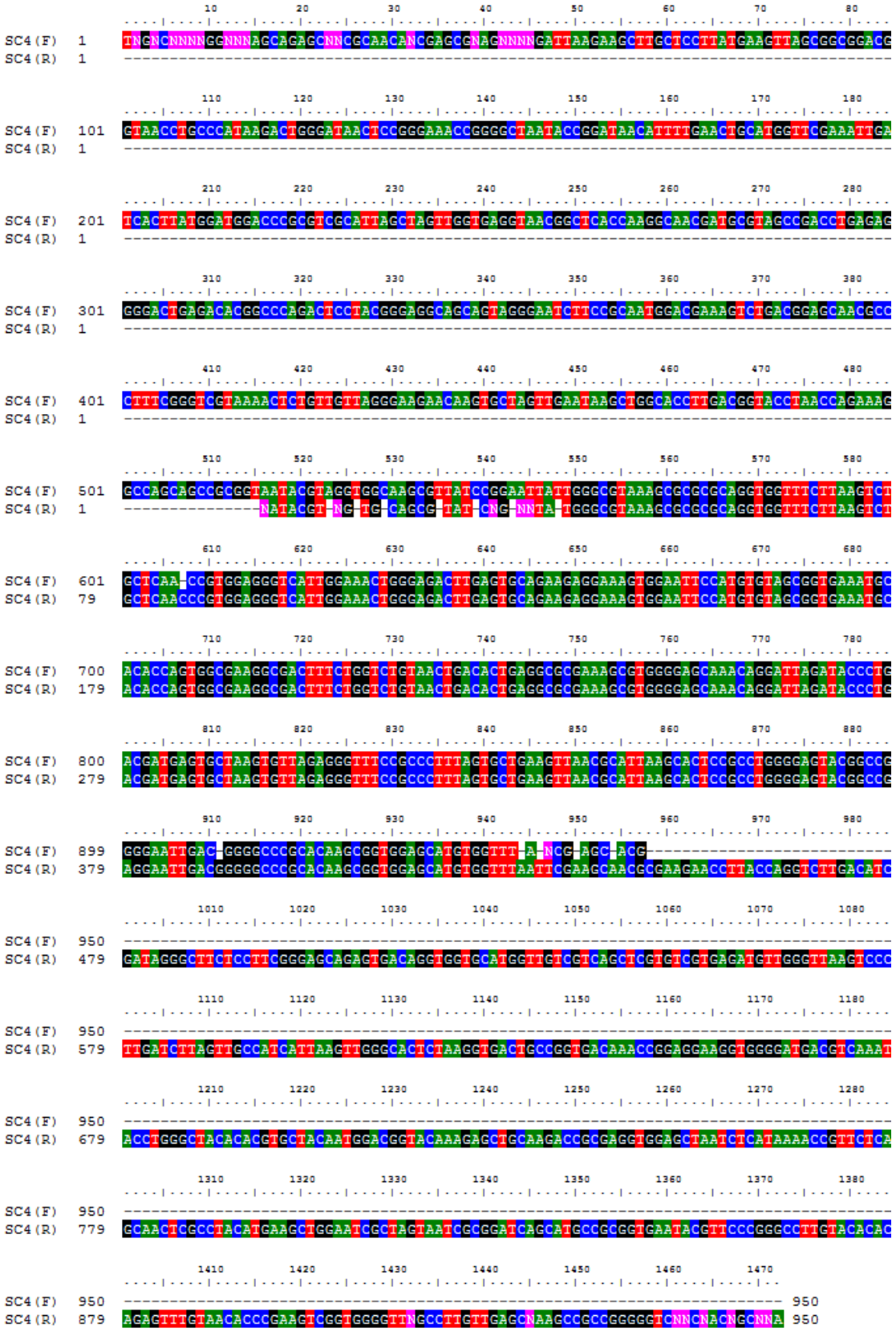

Figure 12B Sequence of 16S rDNA region of isolate SC4 using fD1 and rP2 primers.

1.7 Bacterial isolate CSP2-25-1-1

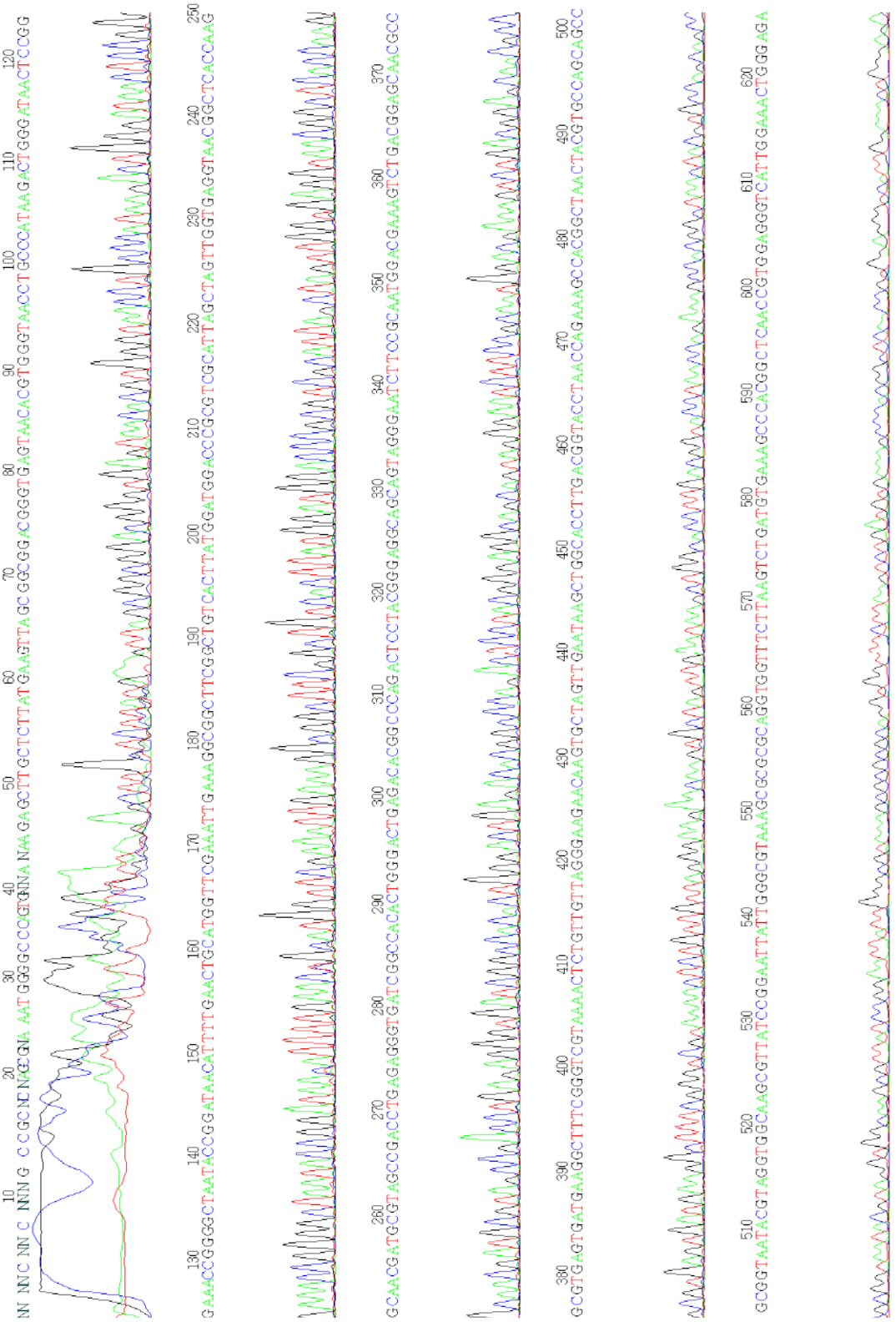

Figure 13B Sequence electrophenogram of 16S rDNA region of isolate CSP2-25-1-1 using fD1 and rP2 primers.

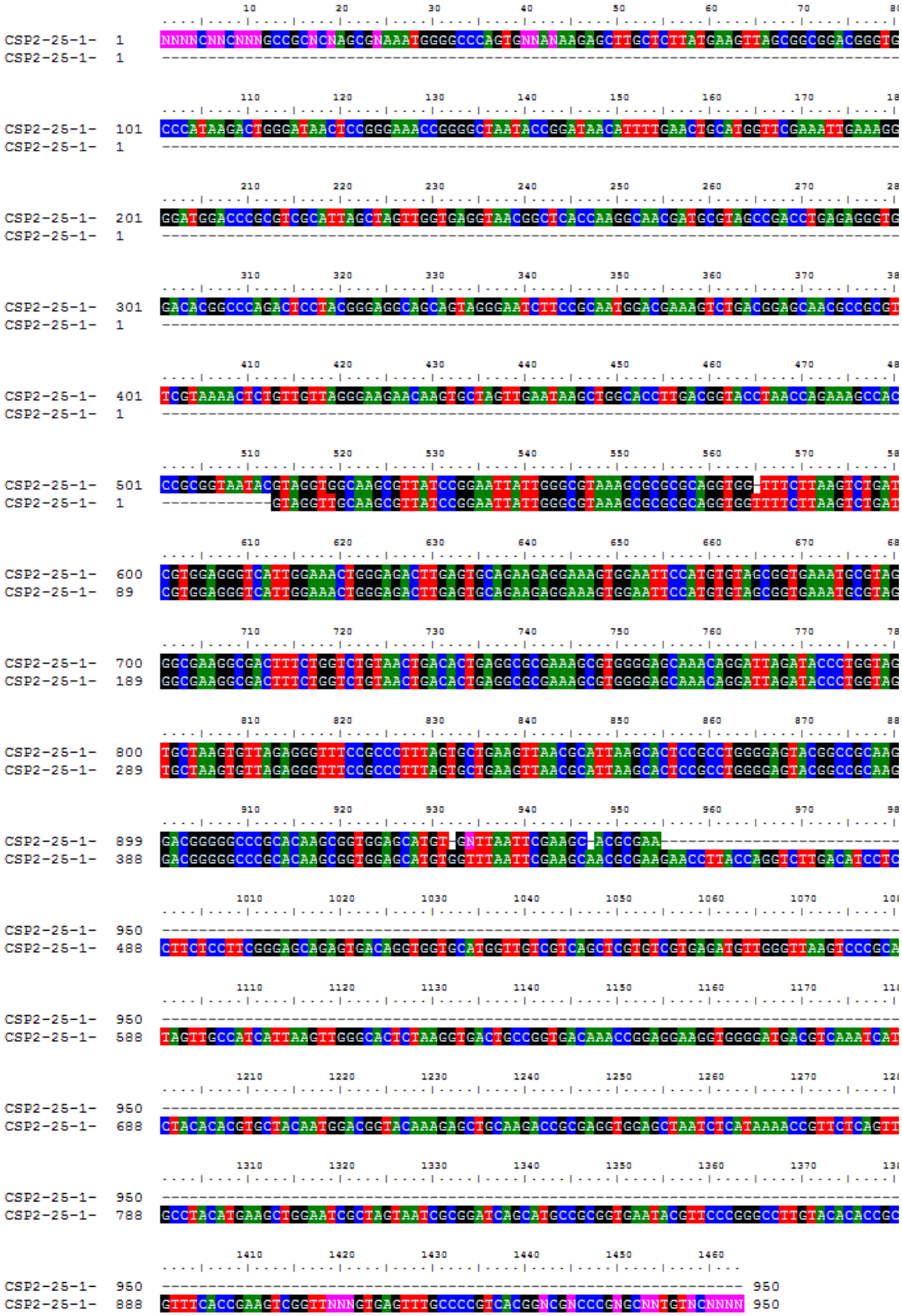

Figure 14B Sequence of 16S rDNA region of isolate CSP2-25-1-1 using fD1 and rP2 primers.

1.8 Bacterial isolate CCA1-24

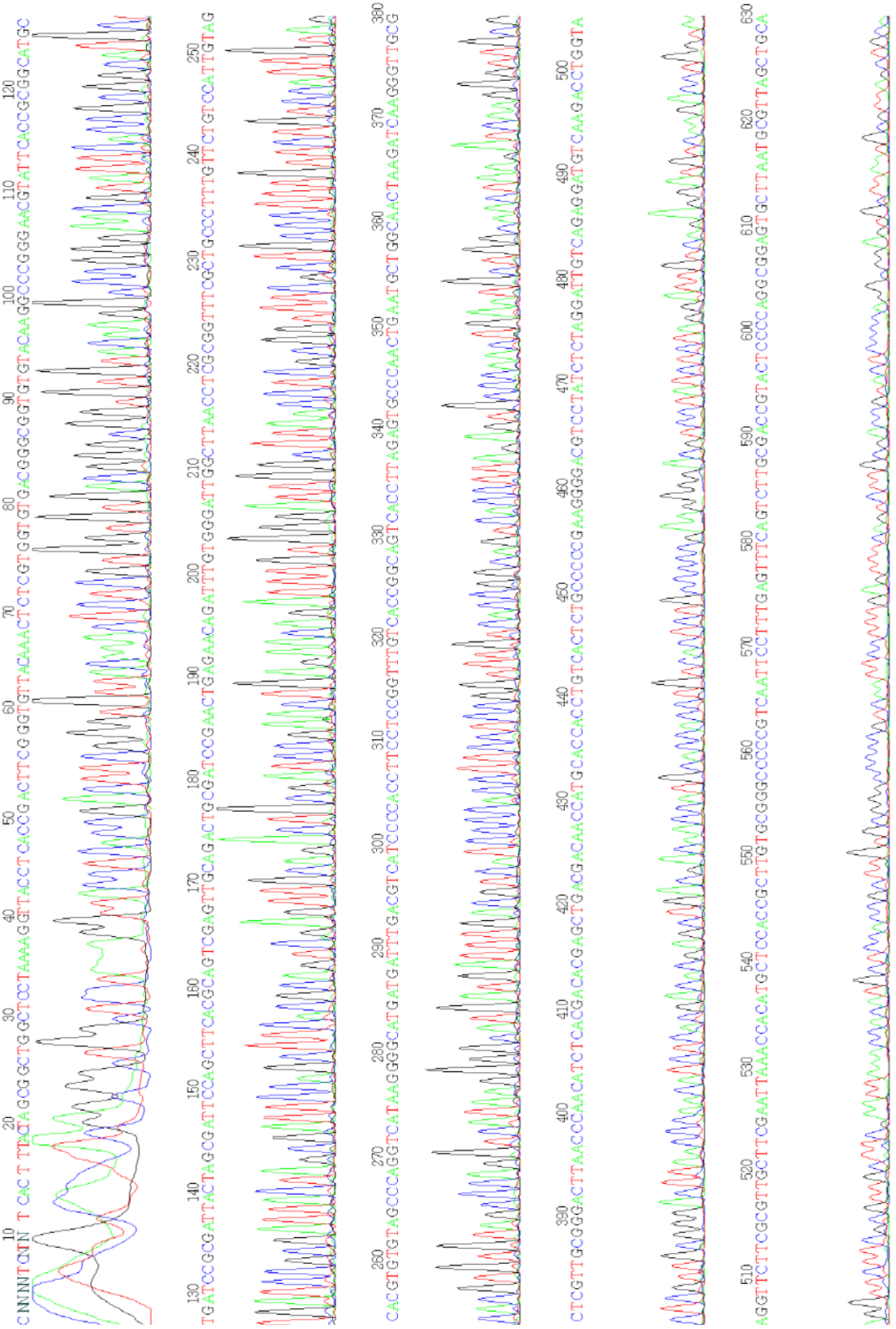

Figure 15B Sequence electrophenogram of 16S rDNA region of isolate CCA1-24 using fD1 and rP2 primers.

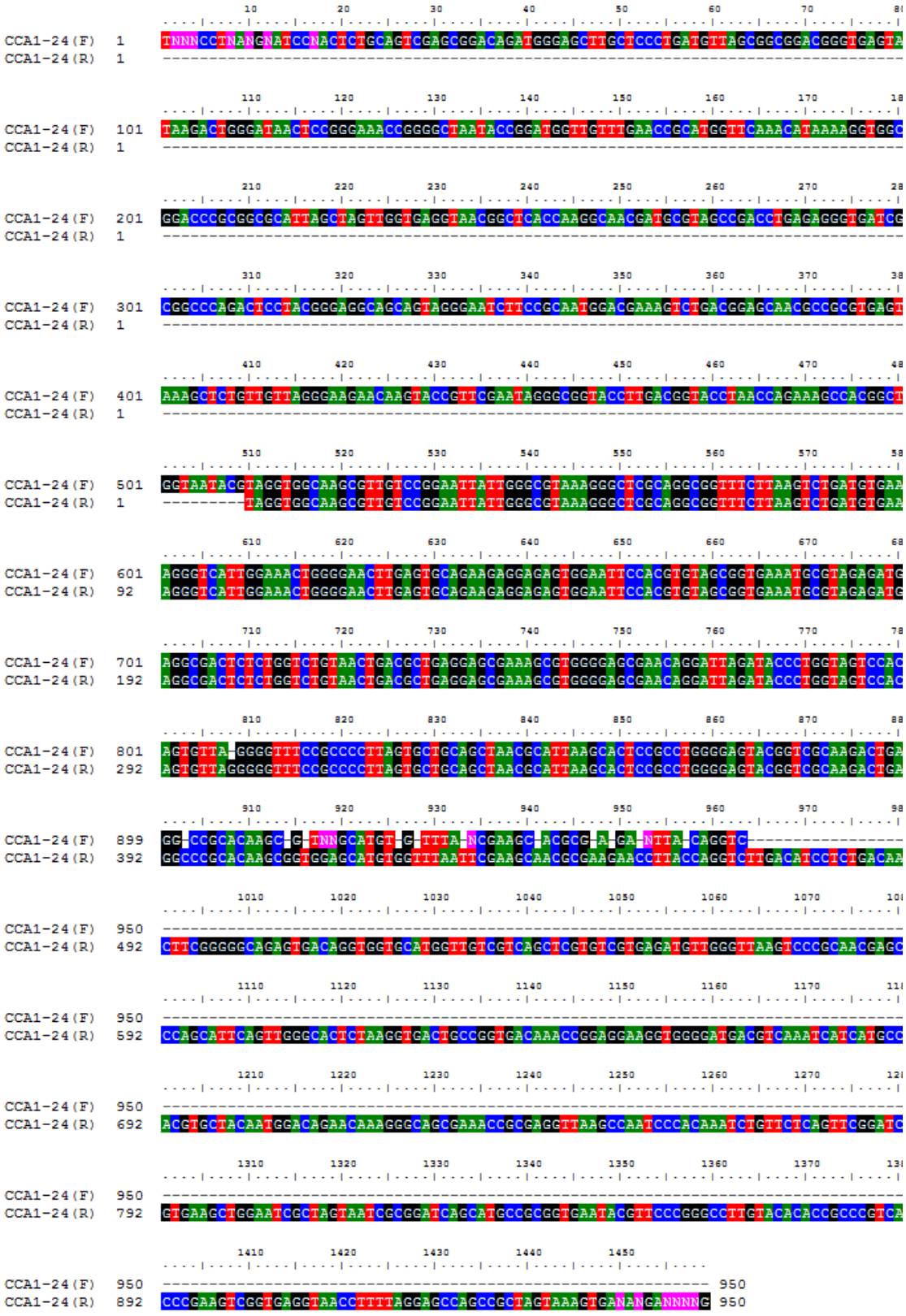

Figure 16B Sequence of 16S rDNA region of isolate CCA1-24 using fD1 and rP2 primers.

1.9 Bacterial isolate CST2-2

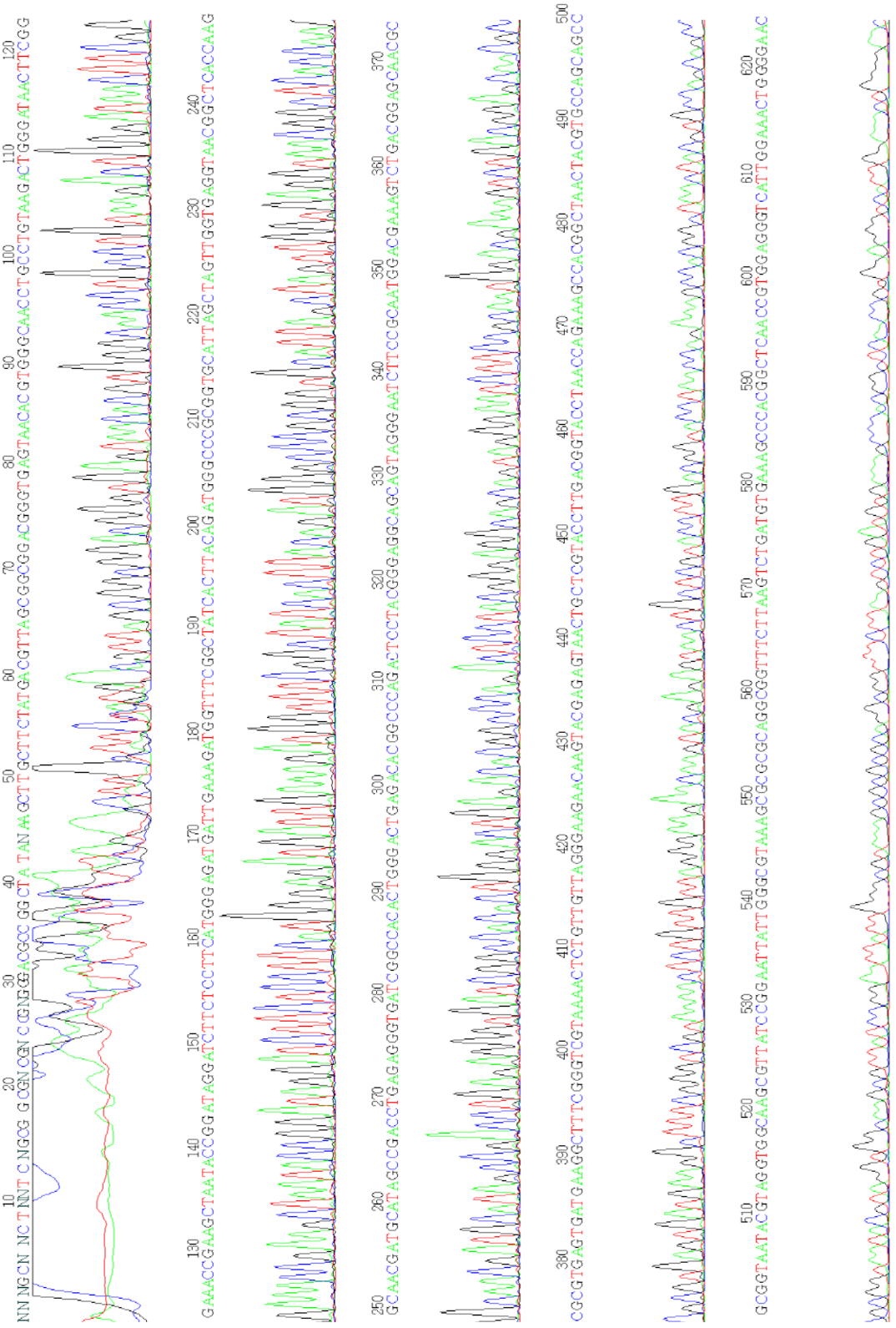

Figure 17B Sequence electrophenogram of 16S rDNA region of isolate CST2-2 using fD1 and rP2 primers.

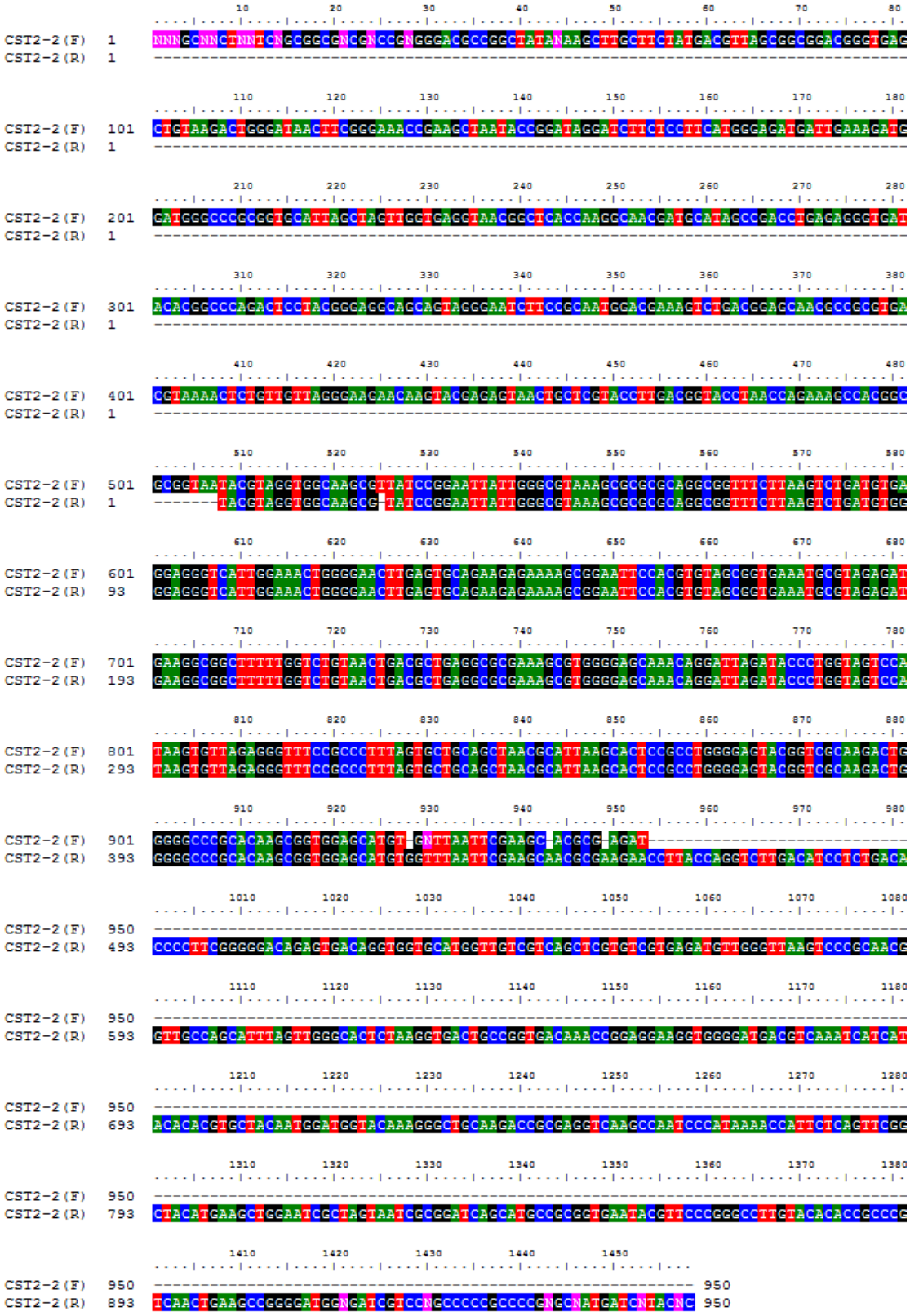

Figure 18B Sequence of 16S rDNA region of isolate CST2-2 using fD1 and rP2 primers.

1.10 Bacterial isolate CCA2-11

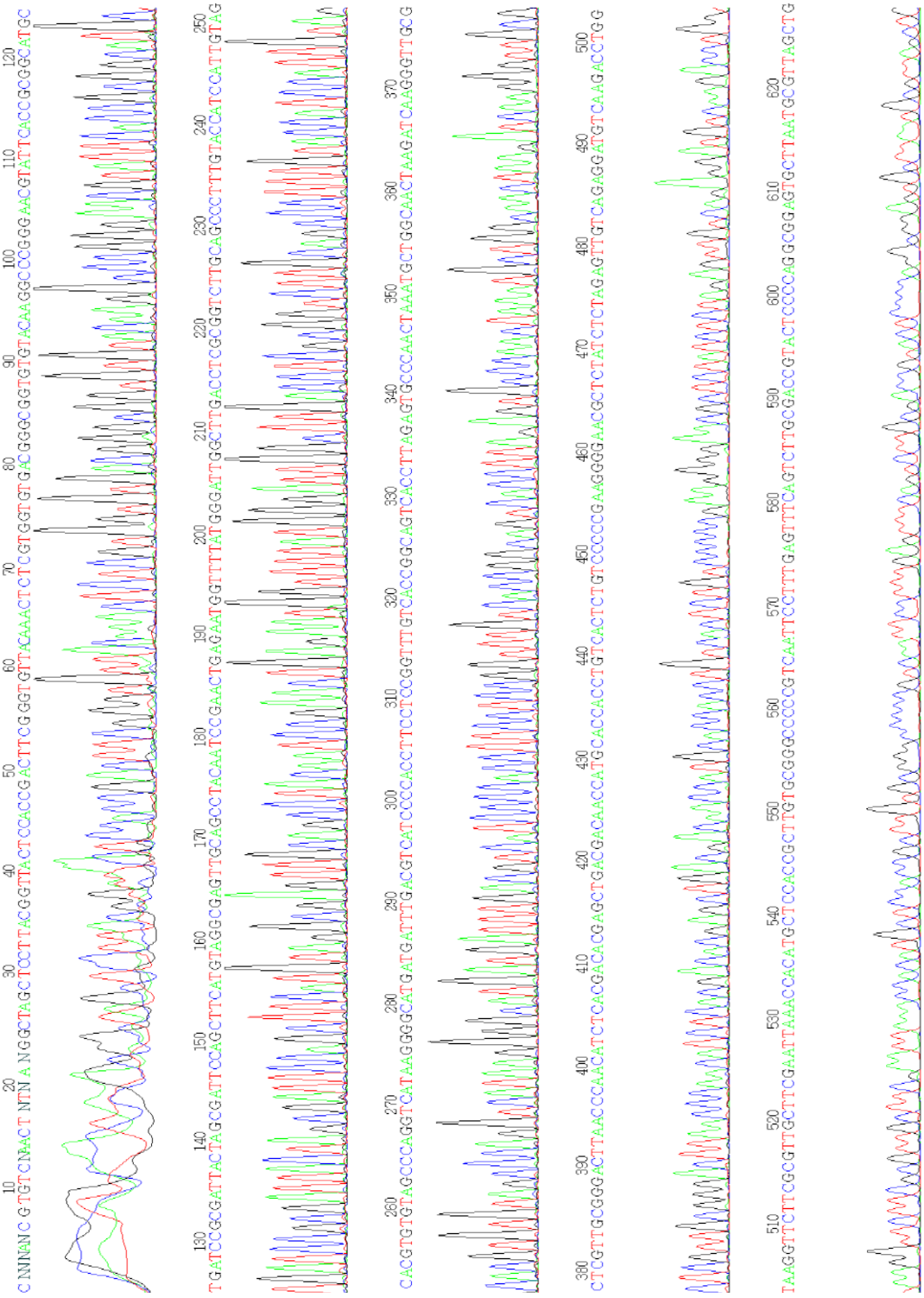

Figure 19B Sequence electrophenogram of isolate 16S rDNA region of CCA2-11 using fD1 and rP2 primers.

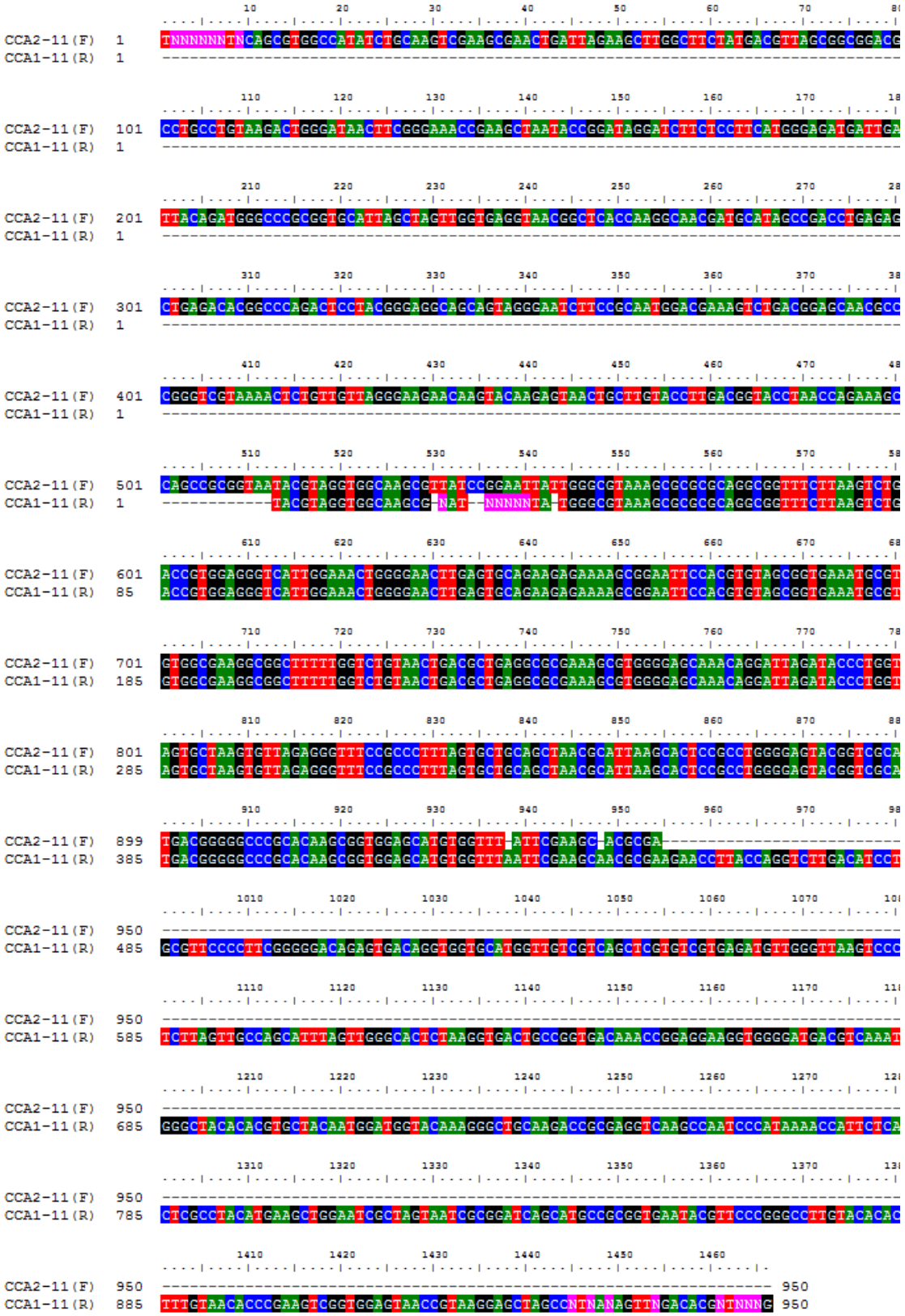

Figure 20B Sequence of 16S rDNA region of isolate CCA2-11 using fD1 and rP2 primers.

1.11 Bacterial isolate CSP2-25-1

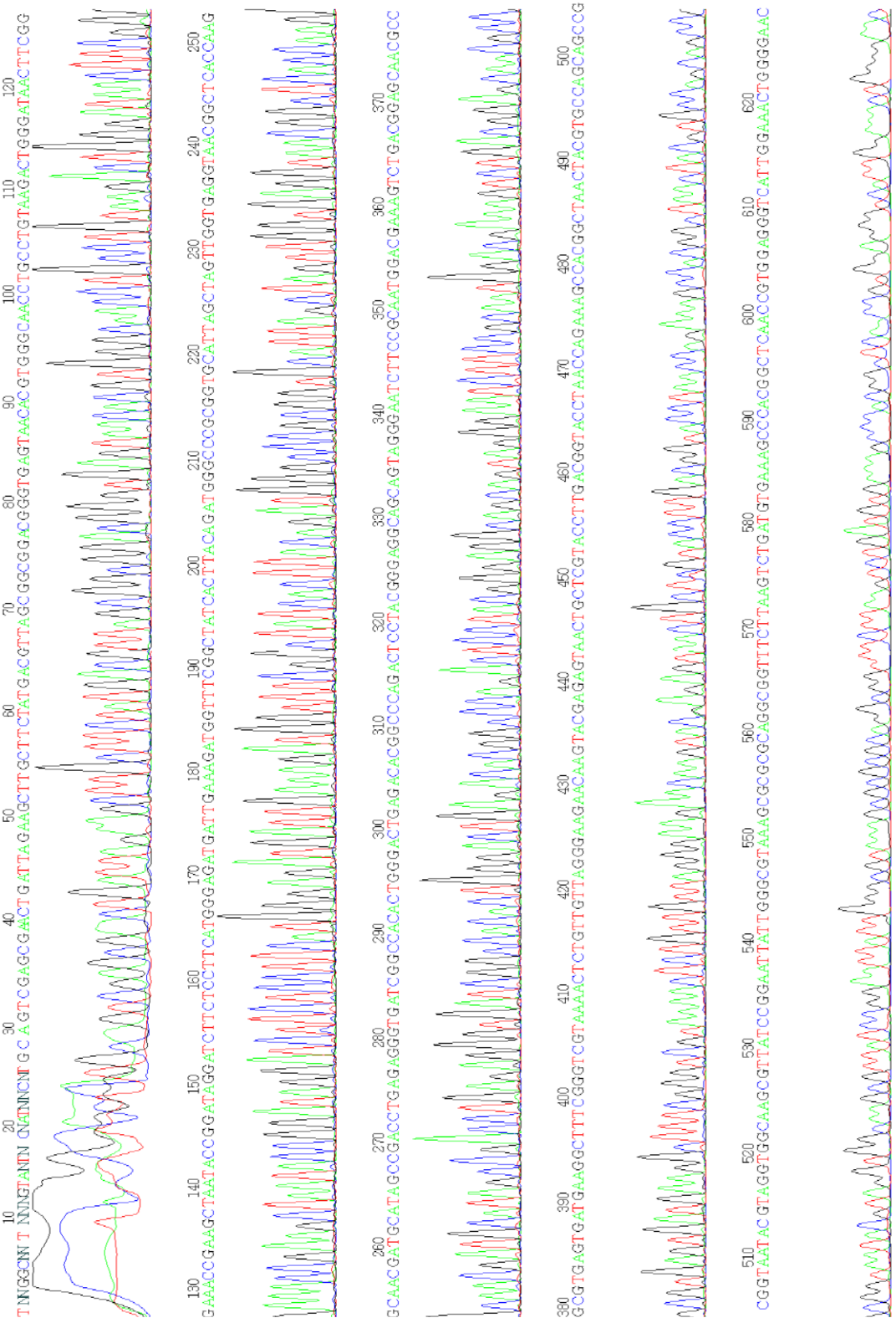

Figure 21B Sequence electrophenogram of 16S rDNA region of isolate CSP2-25-1 using fD1 and rP2 primers.

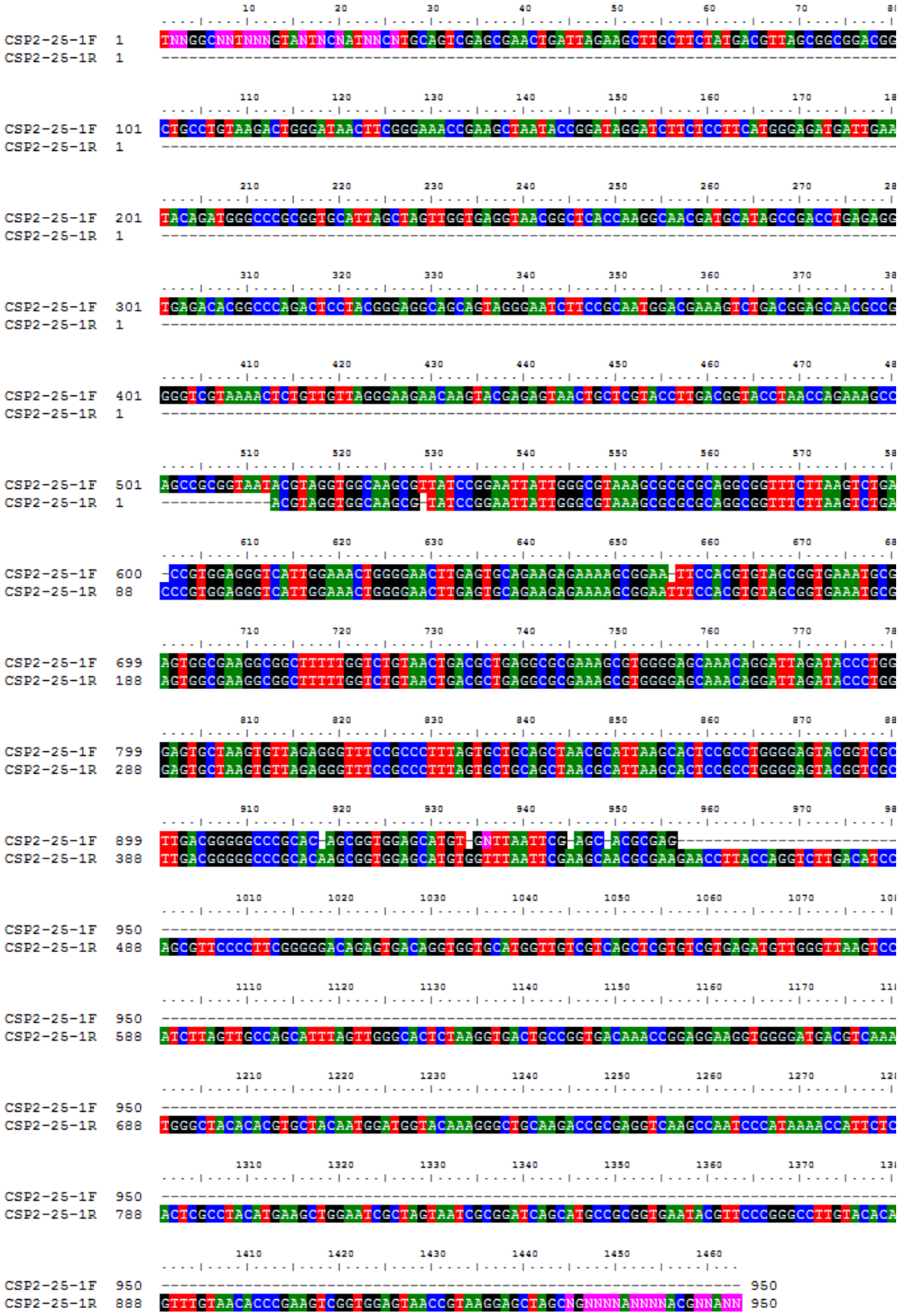

Figure 22B Sequence of 16S rDNA region of isolate CSP2-25-1 using fD1 and rP2 primers.

1.12 Bacterial isolate CWC1-6-1

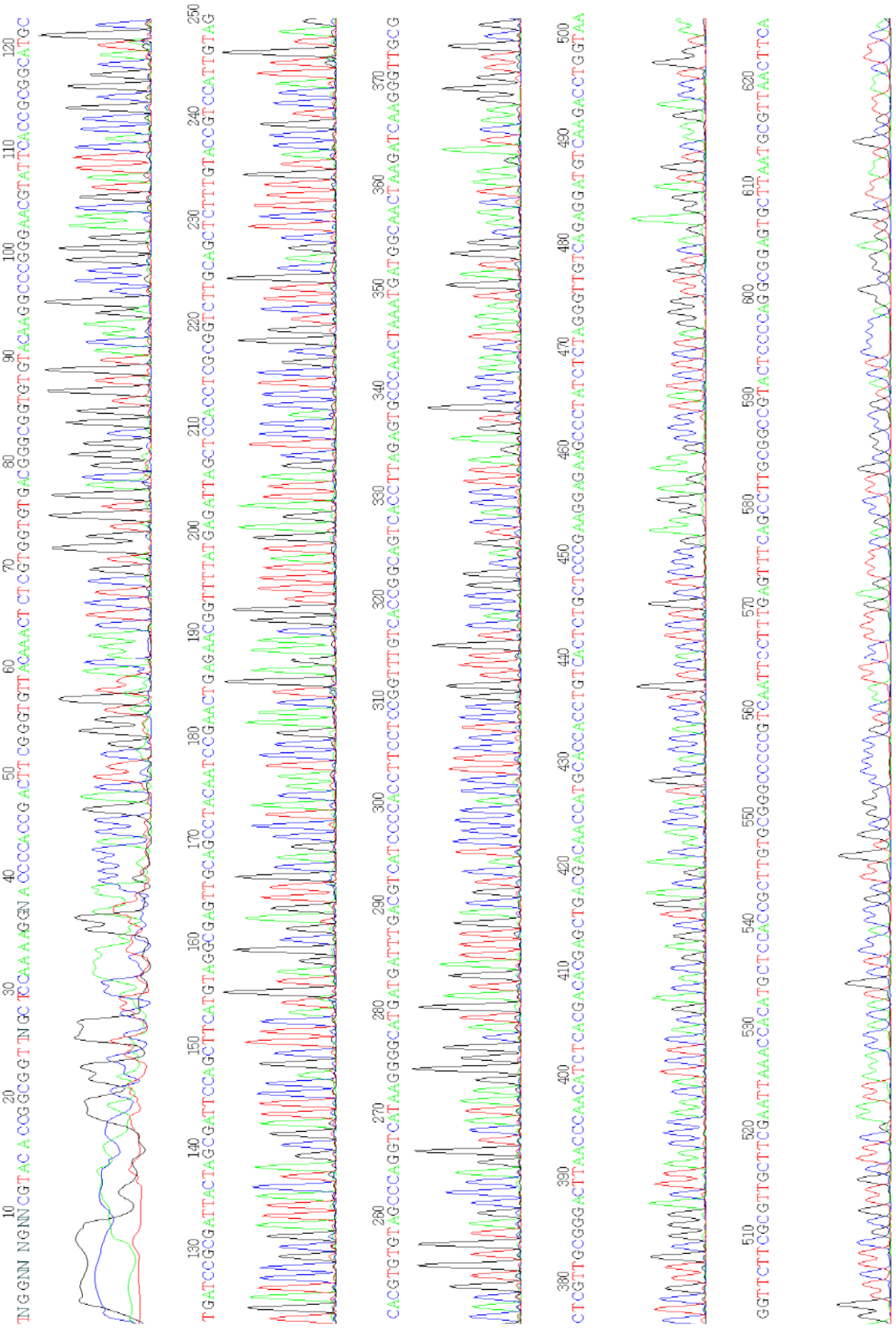

Figure 23B Sequence electrophenogram of 16S rDNA region of isolate CWC1-6-1 using fD1 and rP2 primers.

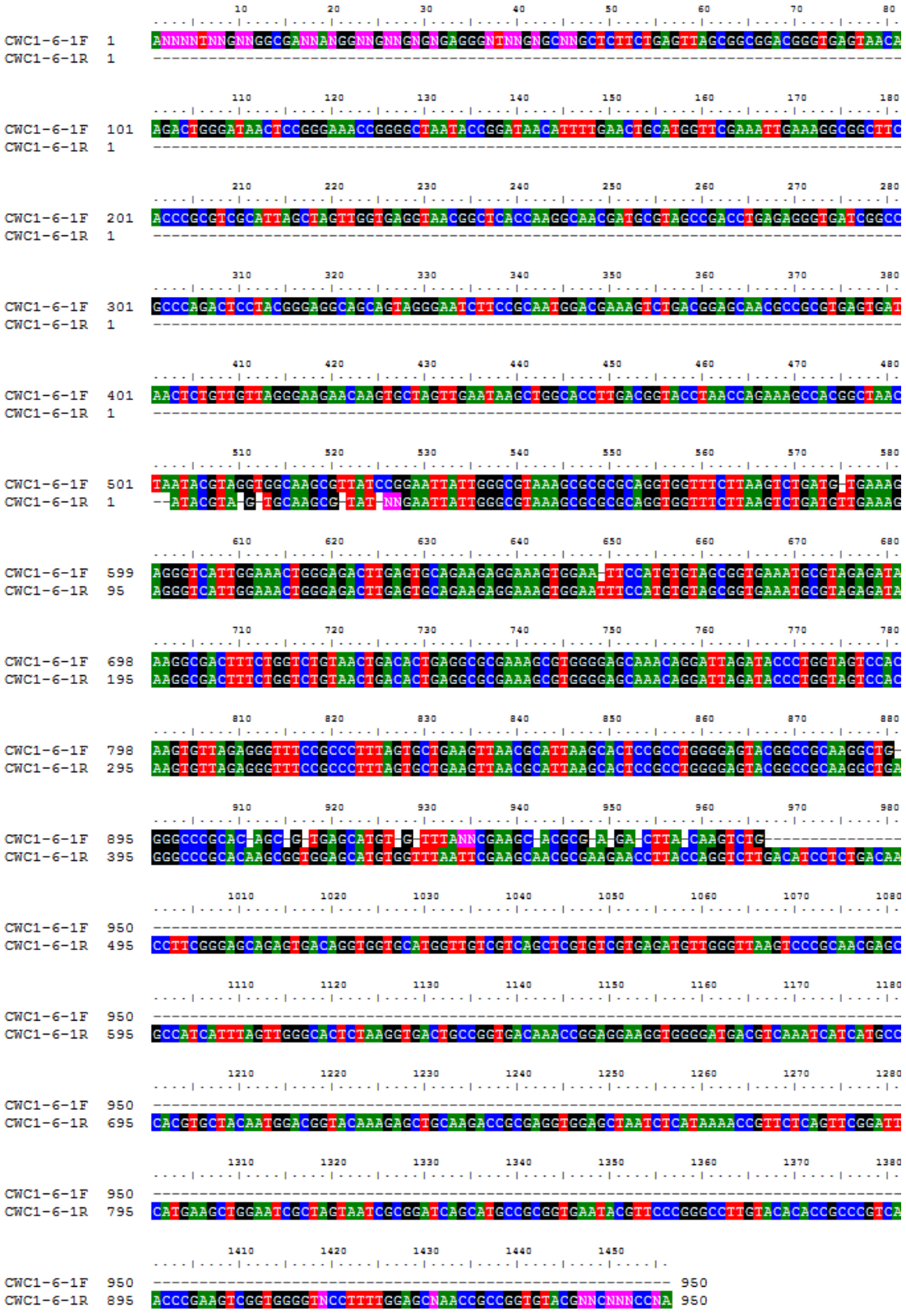

Figure 24B Sequence of 16S rDNA region of isolate CWC1-6-1 using fD1 and rP2 primers.

APPENDIX C

PHYLOGENETIC TREE CONSTRUCTION

1. Alignment of DNA sequences

Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Staphylococcus* sp. CWP2-16, *Klebsiella* sp. CSP2-21, *Pseudomonas* sp. CSP2-3, *Enterobacter* sp. CAS5-1, and *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1 were examined and compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction (Figures 1.1C-1.5C).

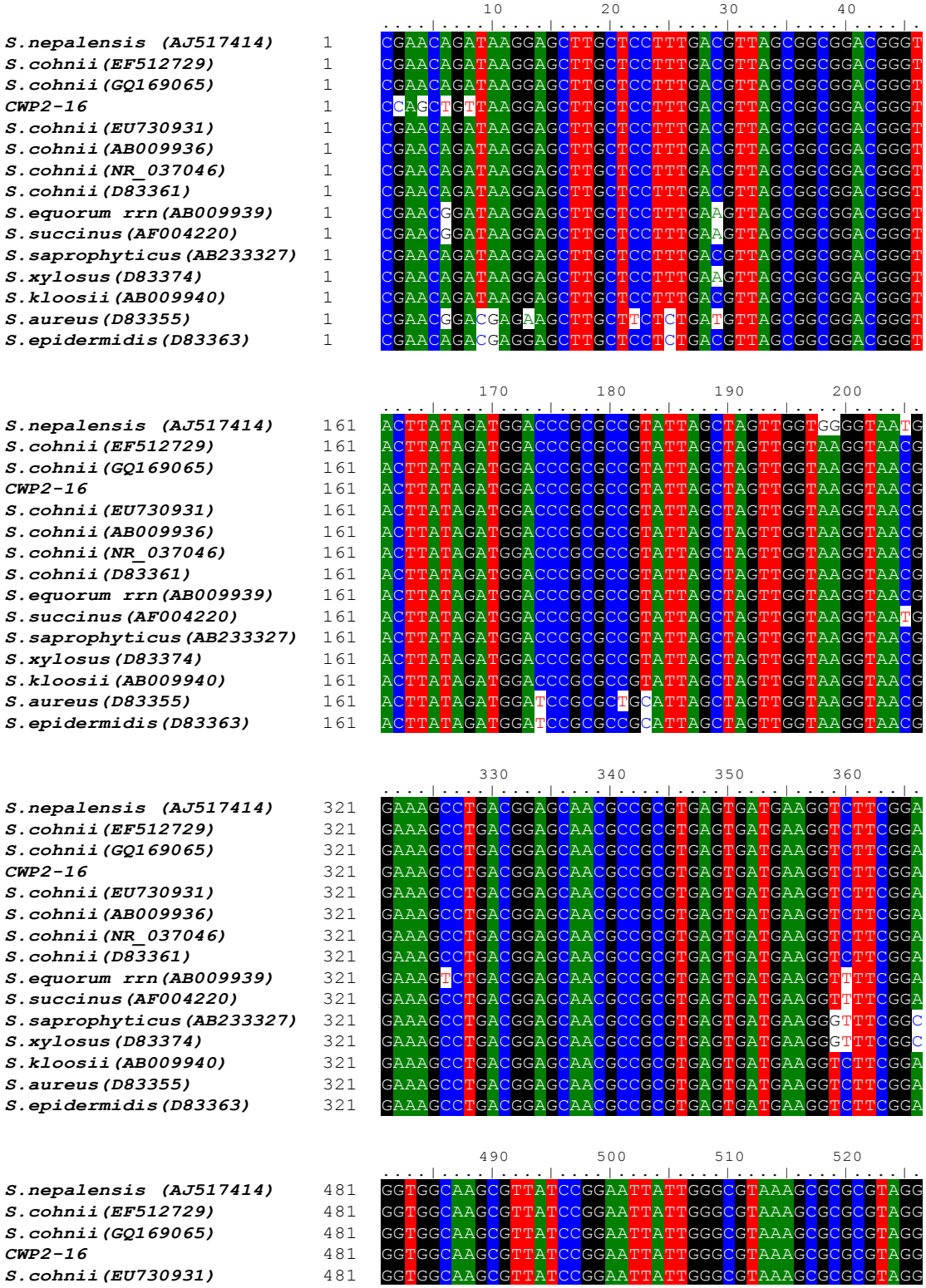

Figure 1.1C Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Staphylococcus* sp. CWP2-16, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

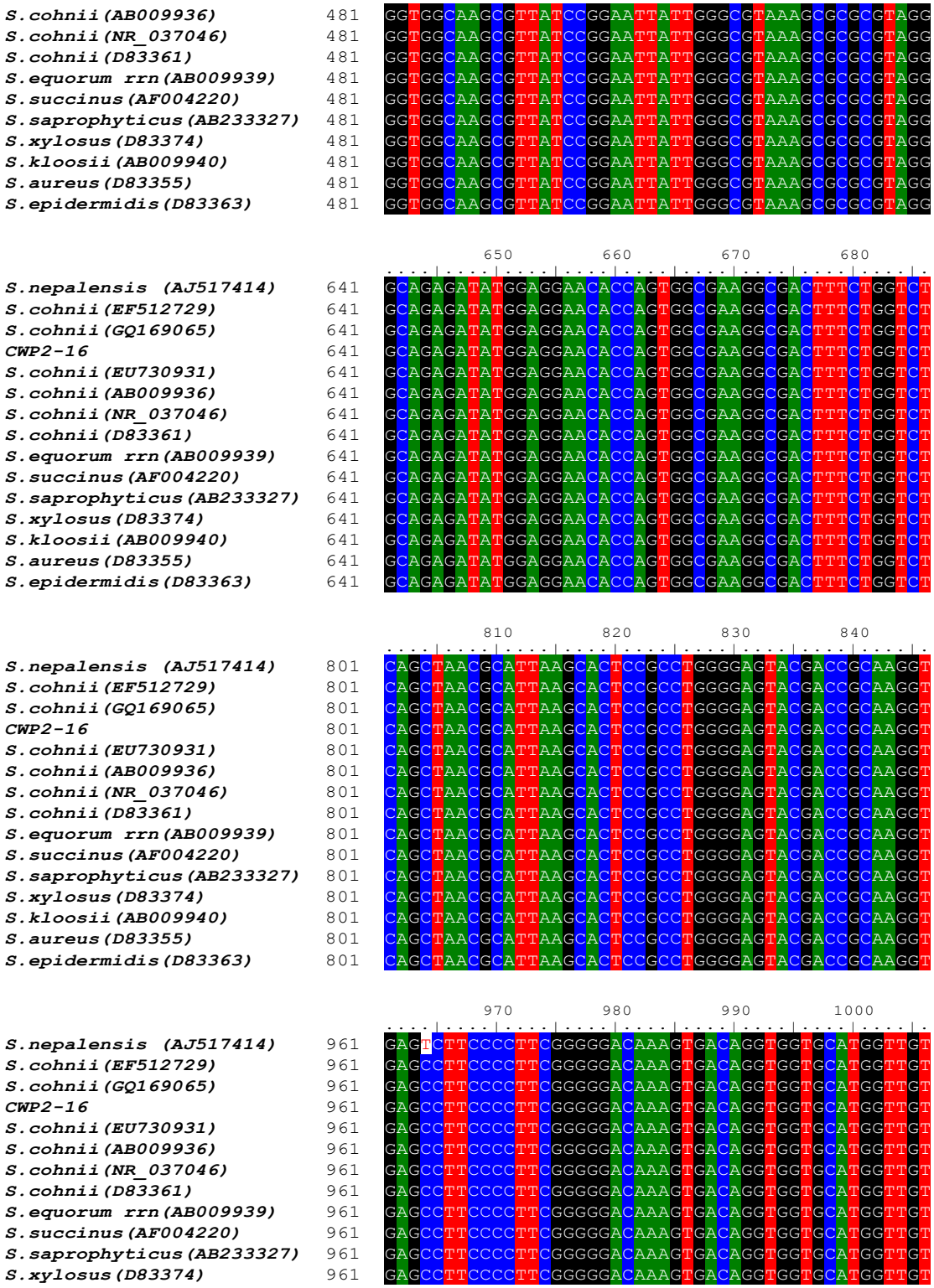

Figure 1.1C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Staphylococcus* sp. CWP2-16, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

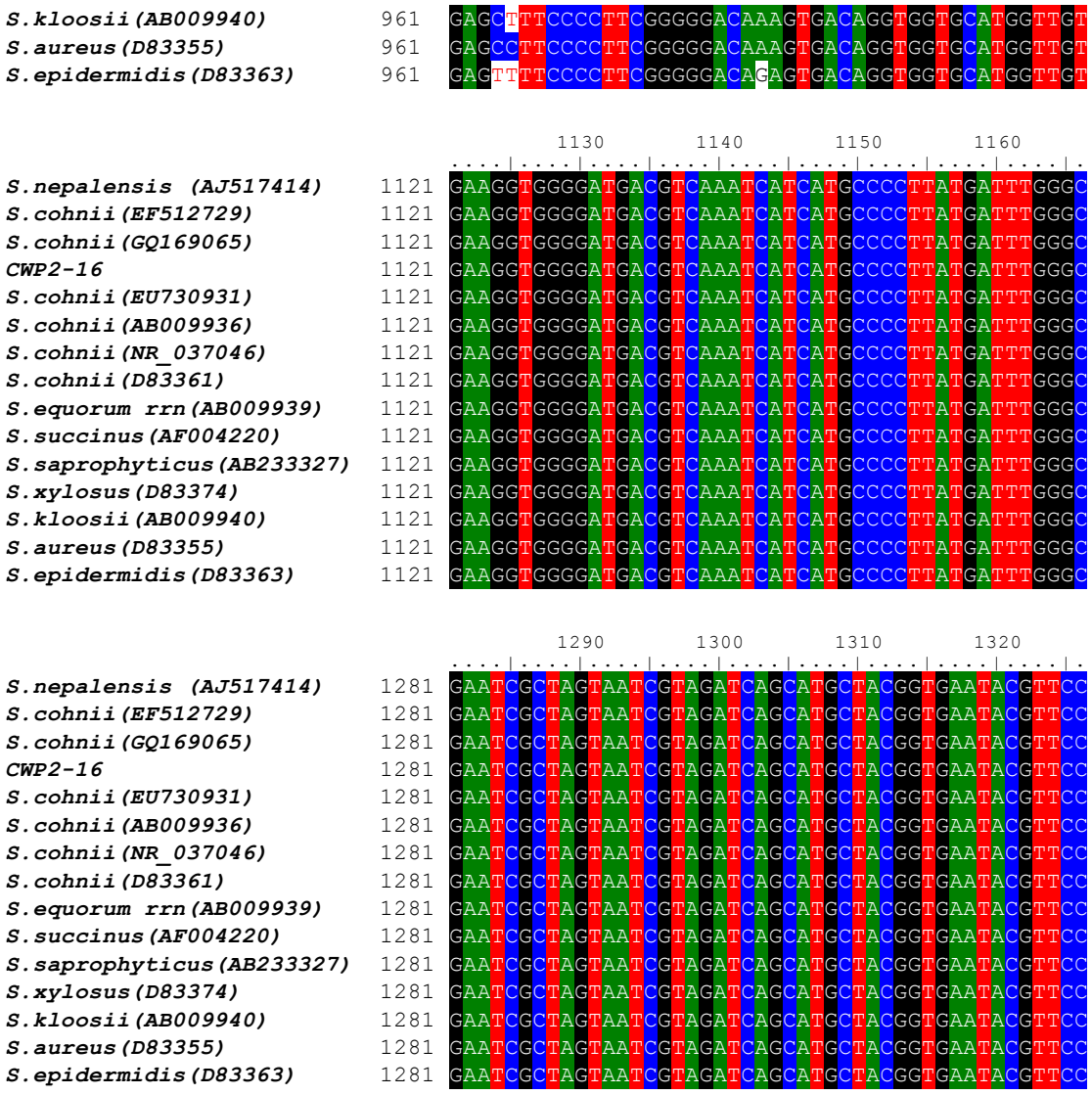

Figure 1.1C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Staphylococcus* sp. CWP2-16, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

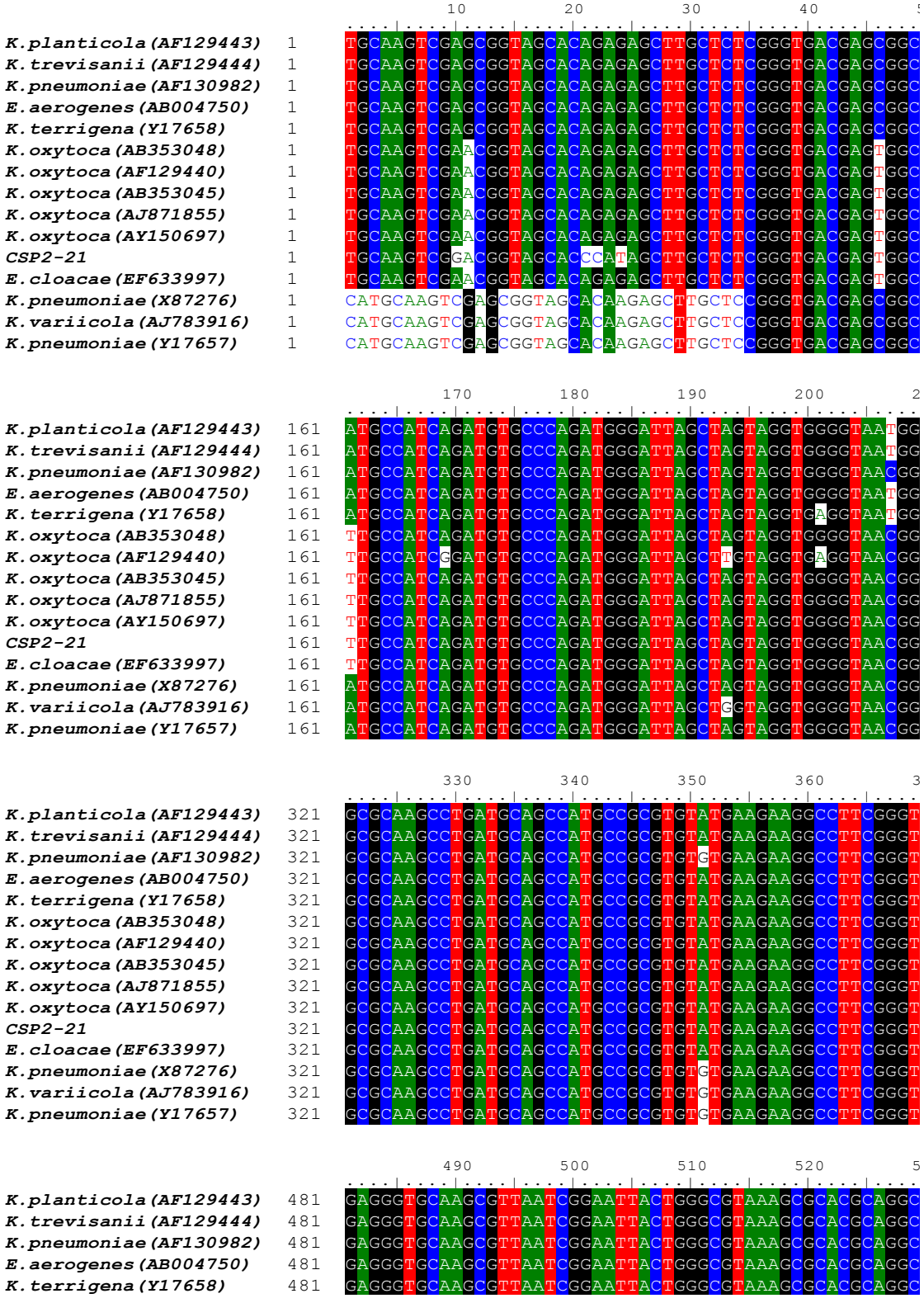

Figure 1.2C Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Klebsiella* sp. CSP2-21, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

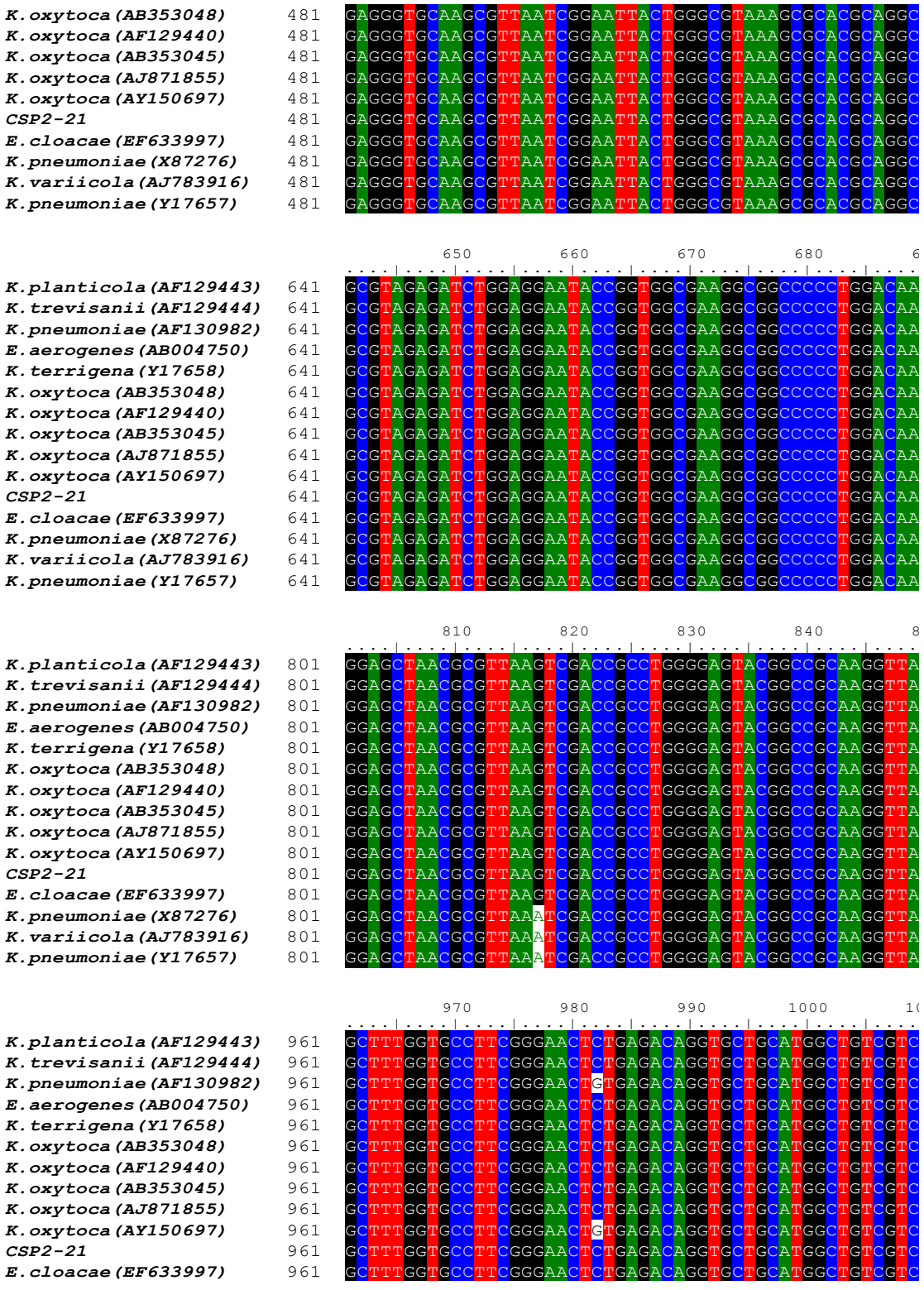

Figure 1.2C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Klebsiella* sp. CSP2-21, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

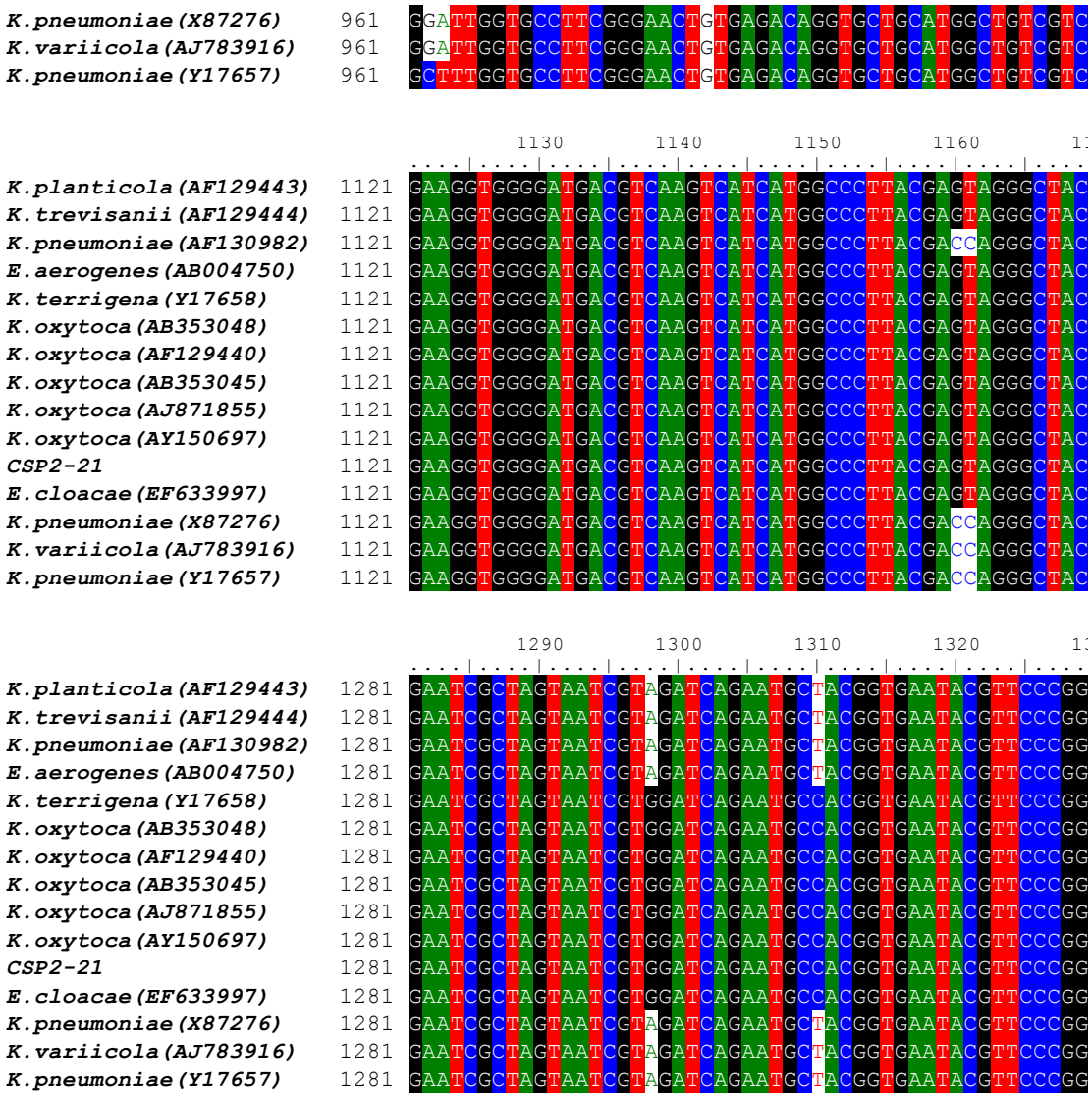

Figure 1.2C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Klebsiella* sp. CSP2-21, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

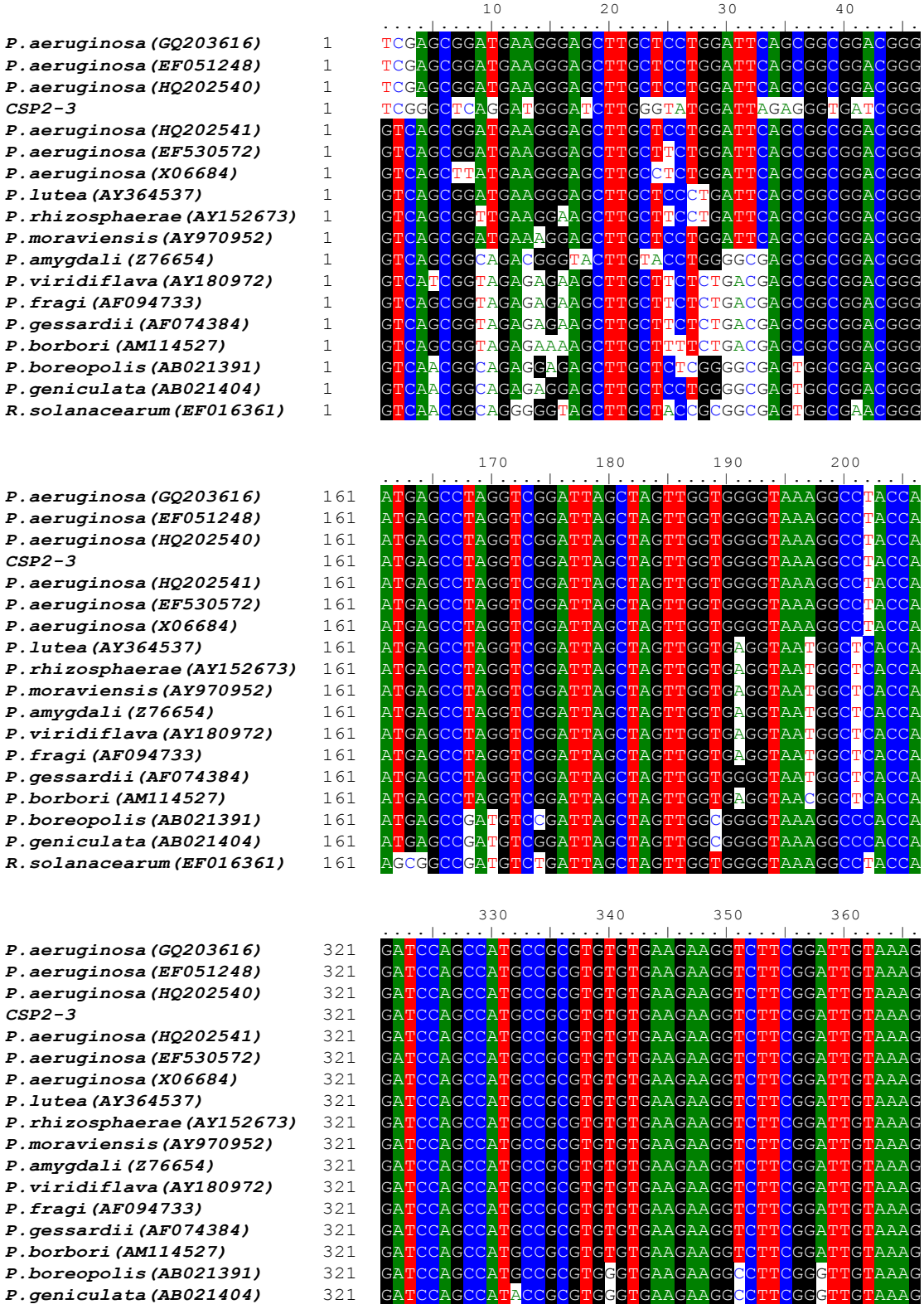

Figure 1.3C Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Pseudomonas* sp. CSP2-3, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

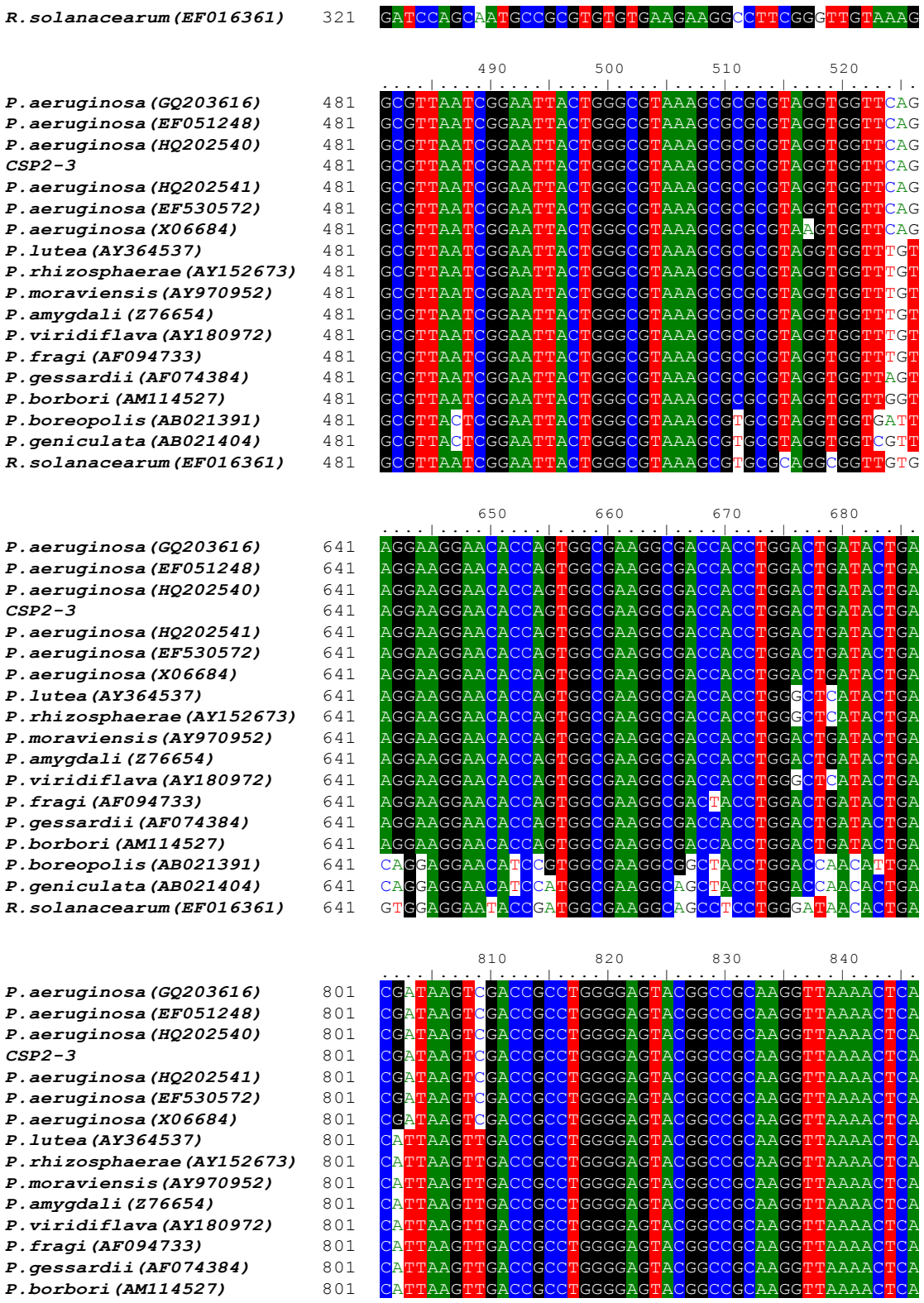

Figure 1.3C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Pseudomonas* sp. CSP2-3, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

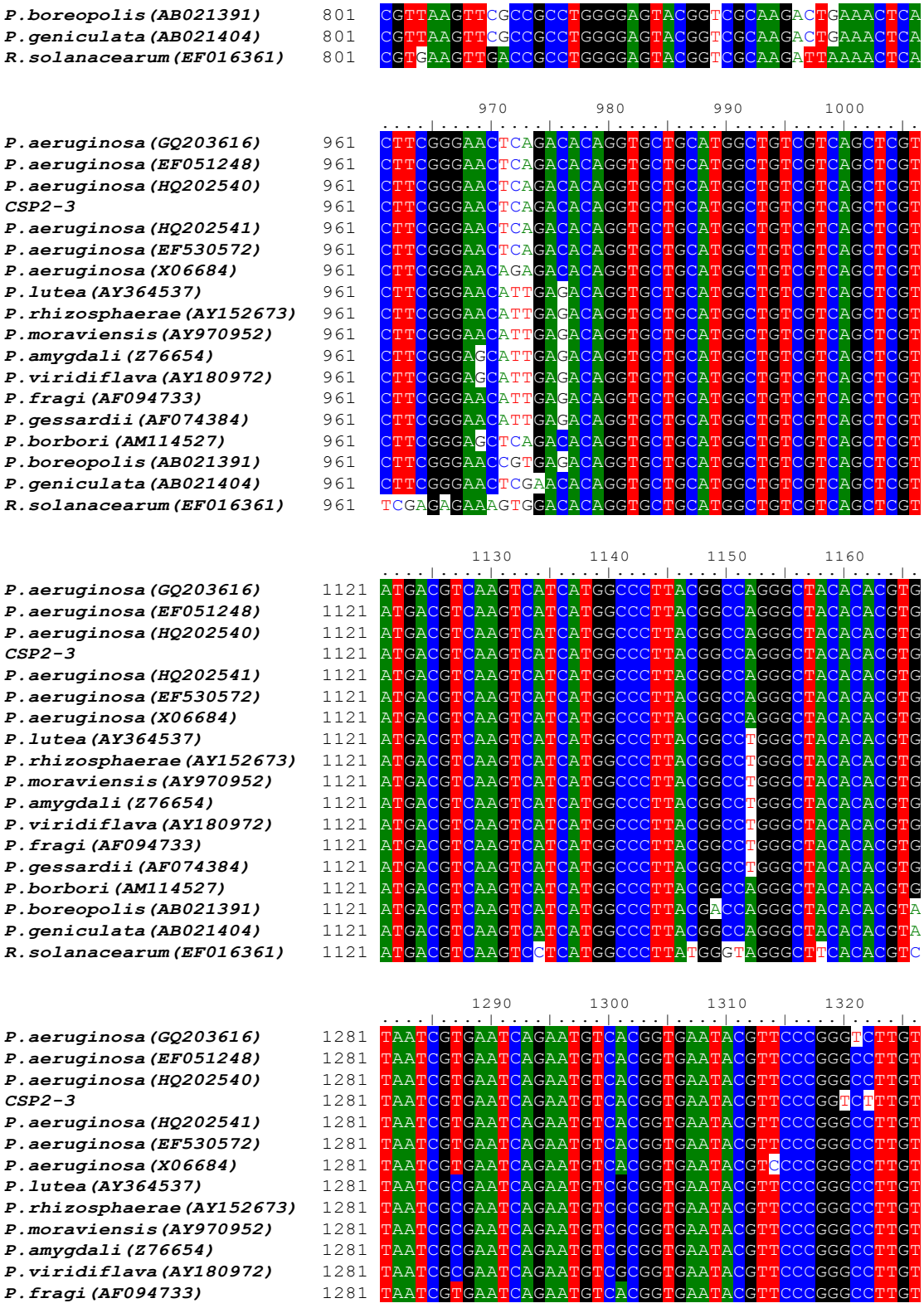

Figure 1.3C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Pseudomonas* sp. CSP2-3, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

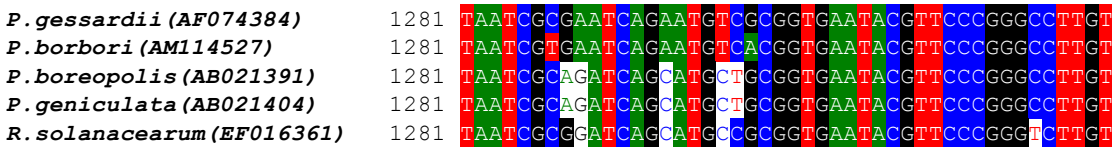

Figure 1.3C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Pseudomonas* sp. CSP2-3, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

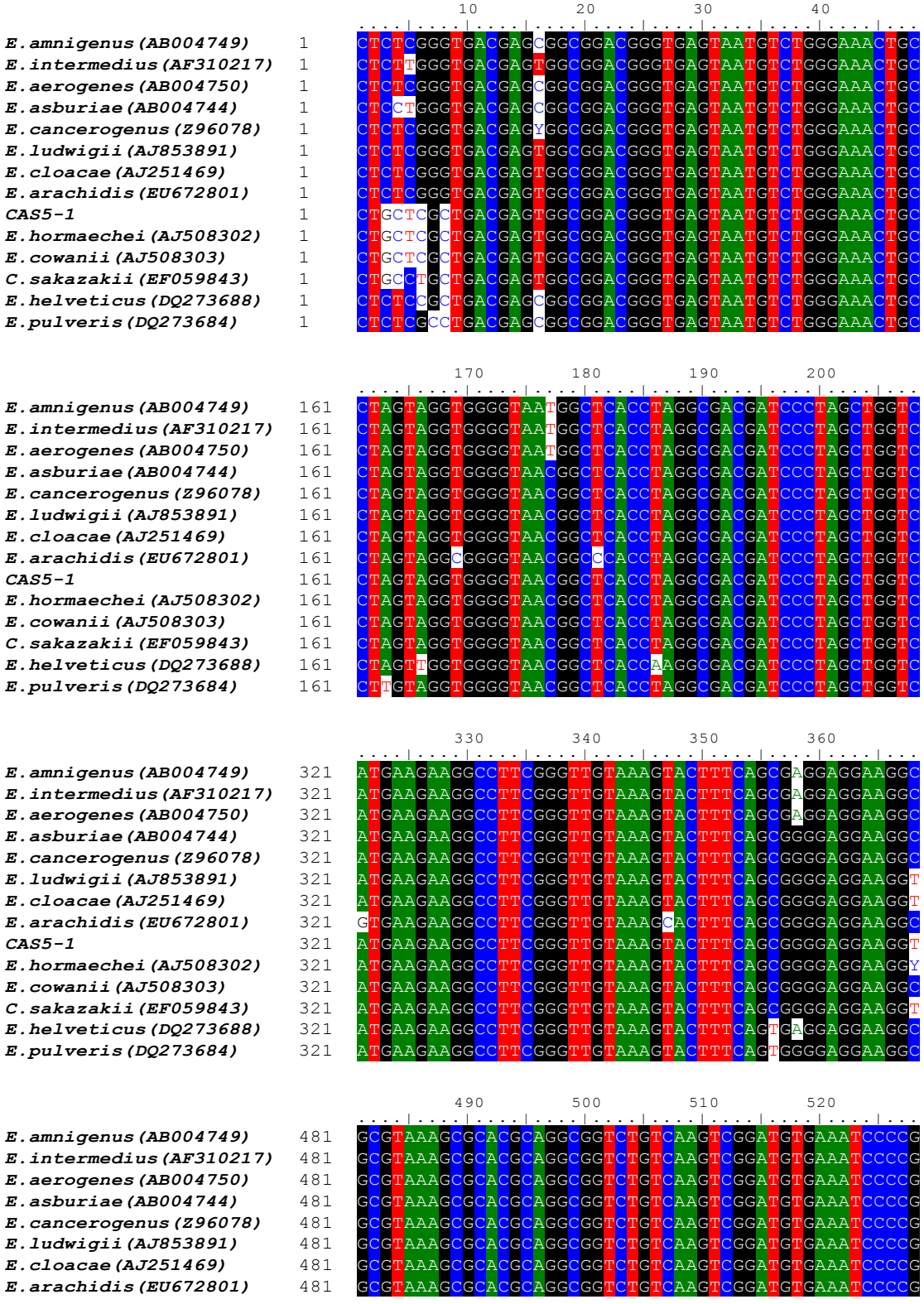

Figure 1.4 C Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Enterobacter* sp. CAS5-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

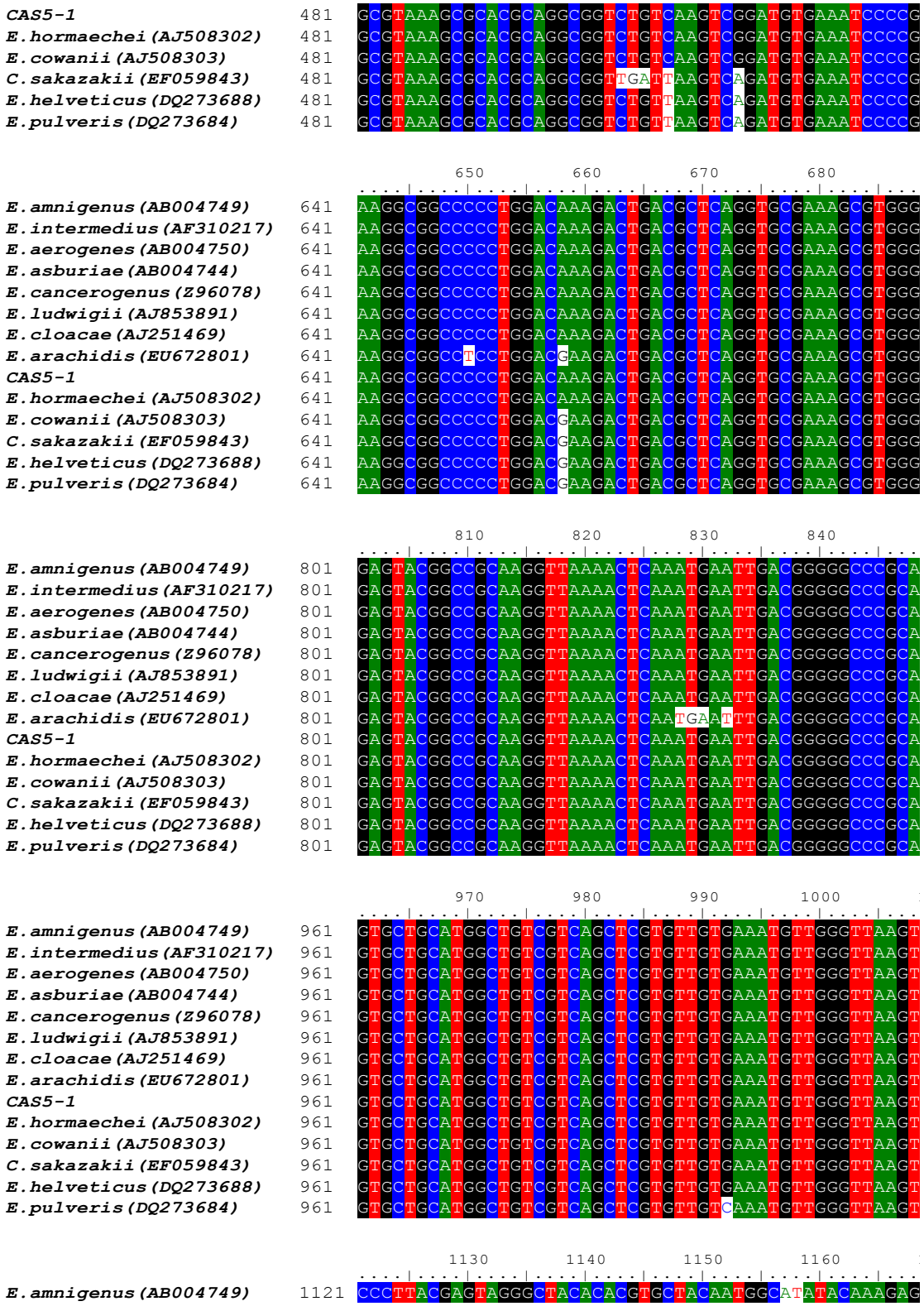

Figure 1.4 C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Enterobacter* sp. CAS5-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

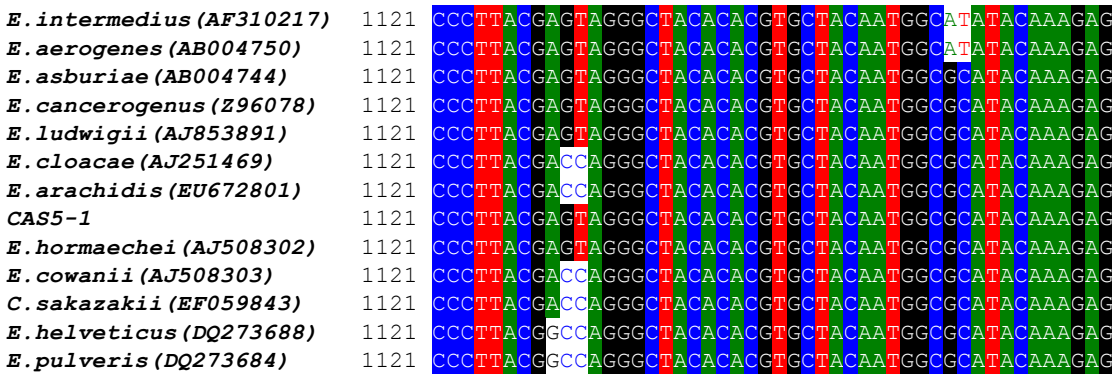

Figure 1.4 C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Enterobacter* sp. CAS5-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

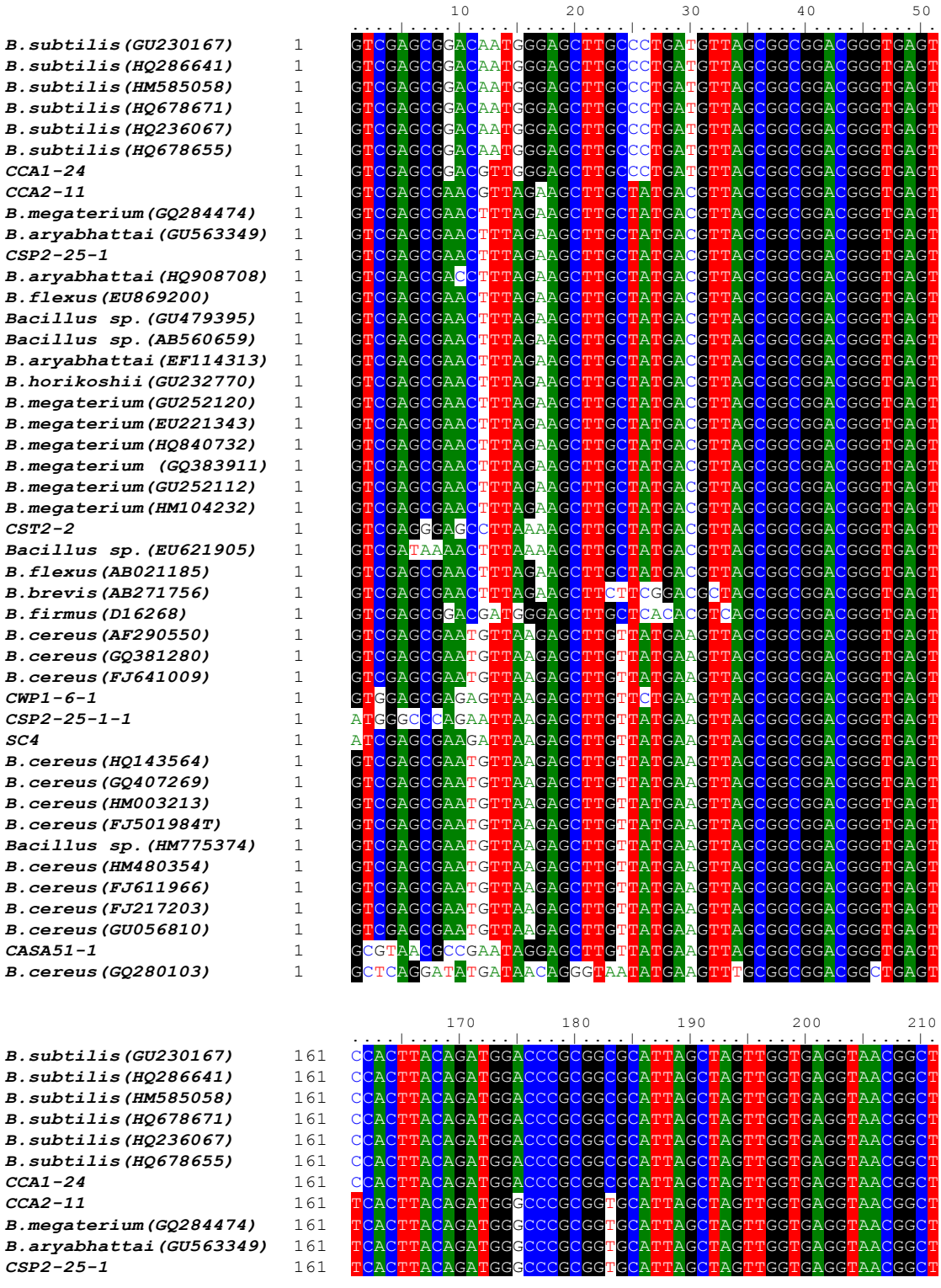

Figure 1.5C Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

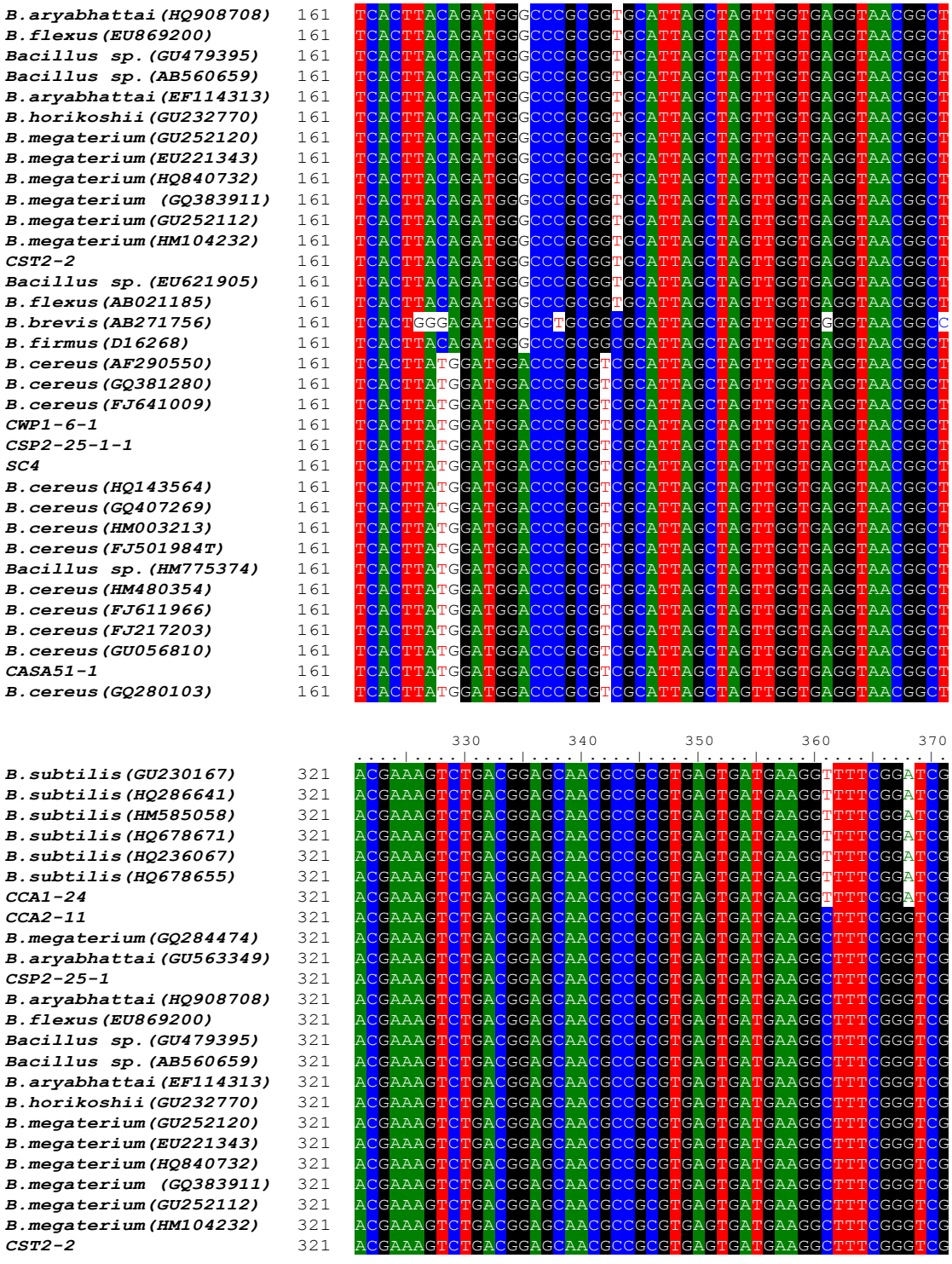

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

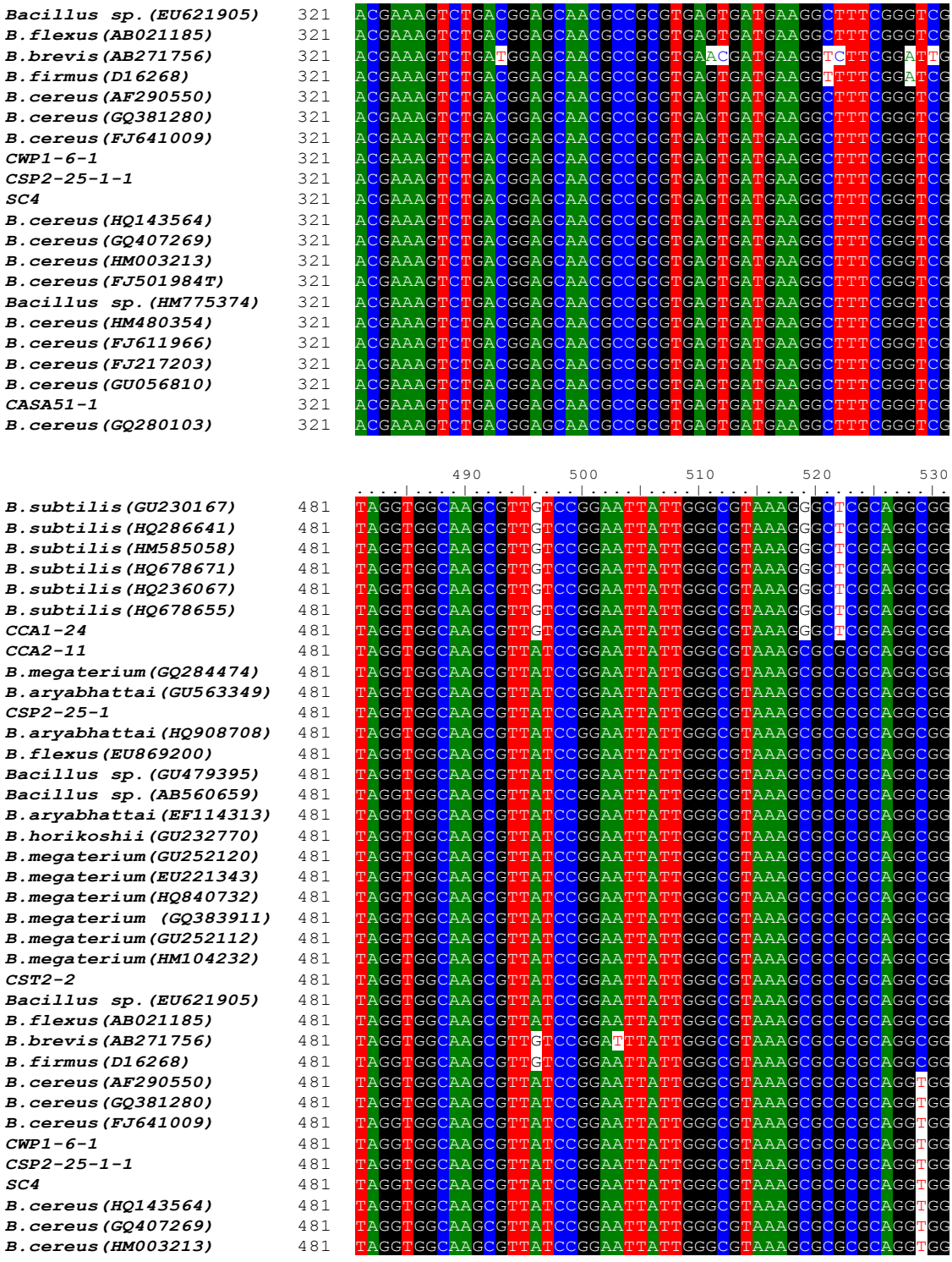

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

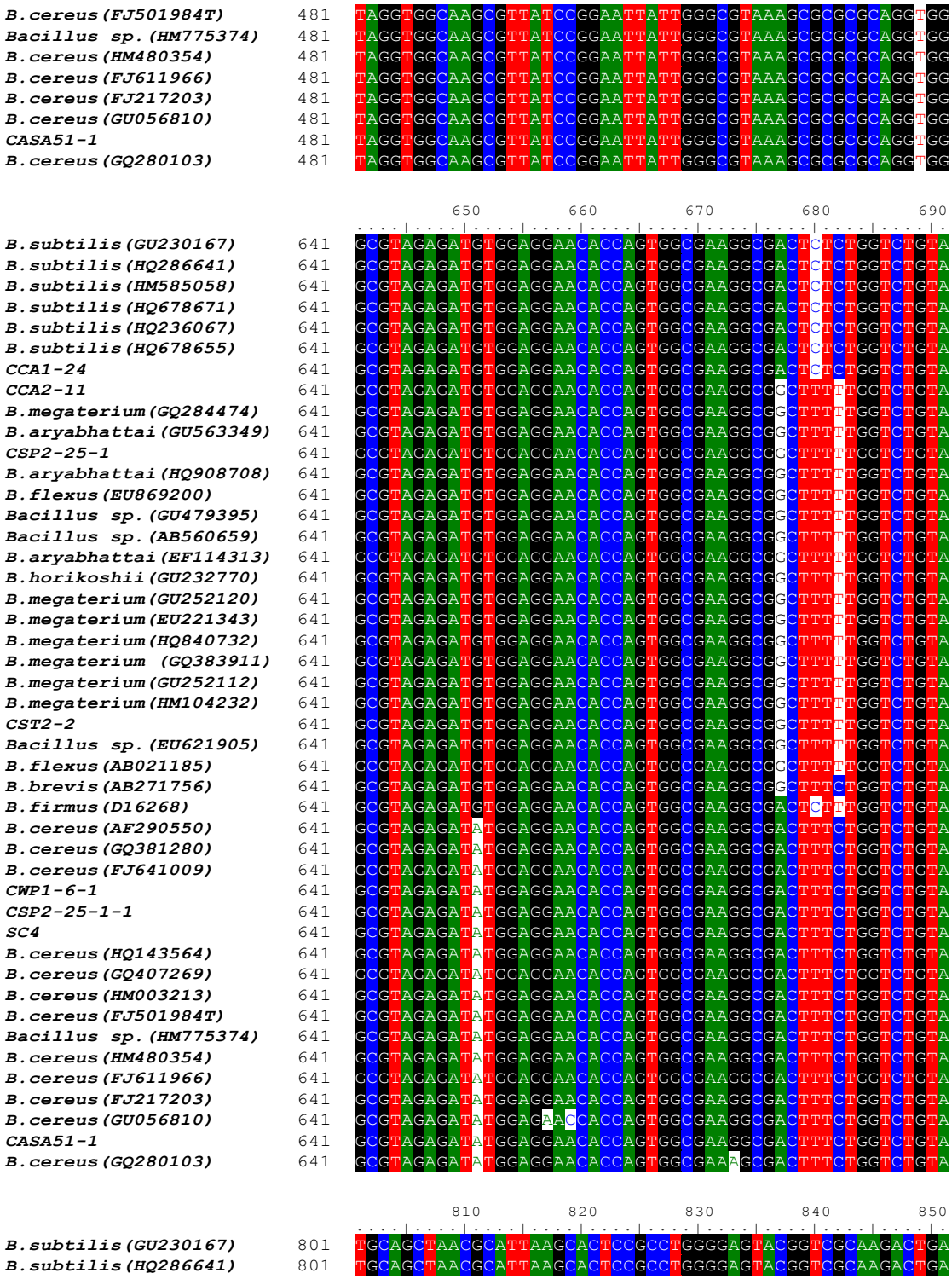

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

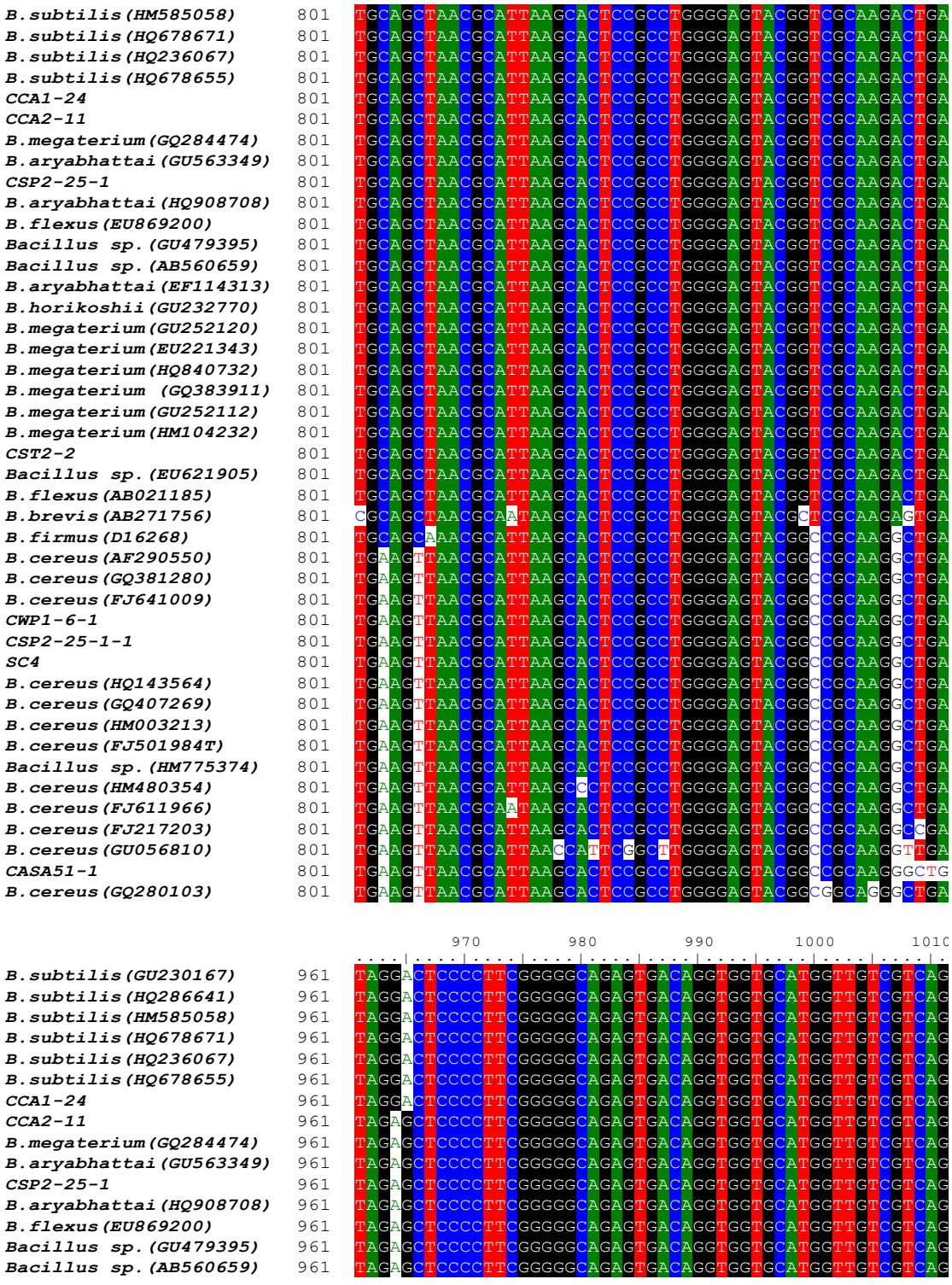

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

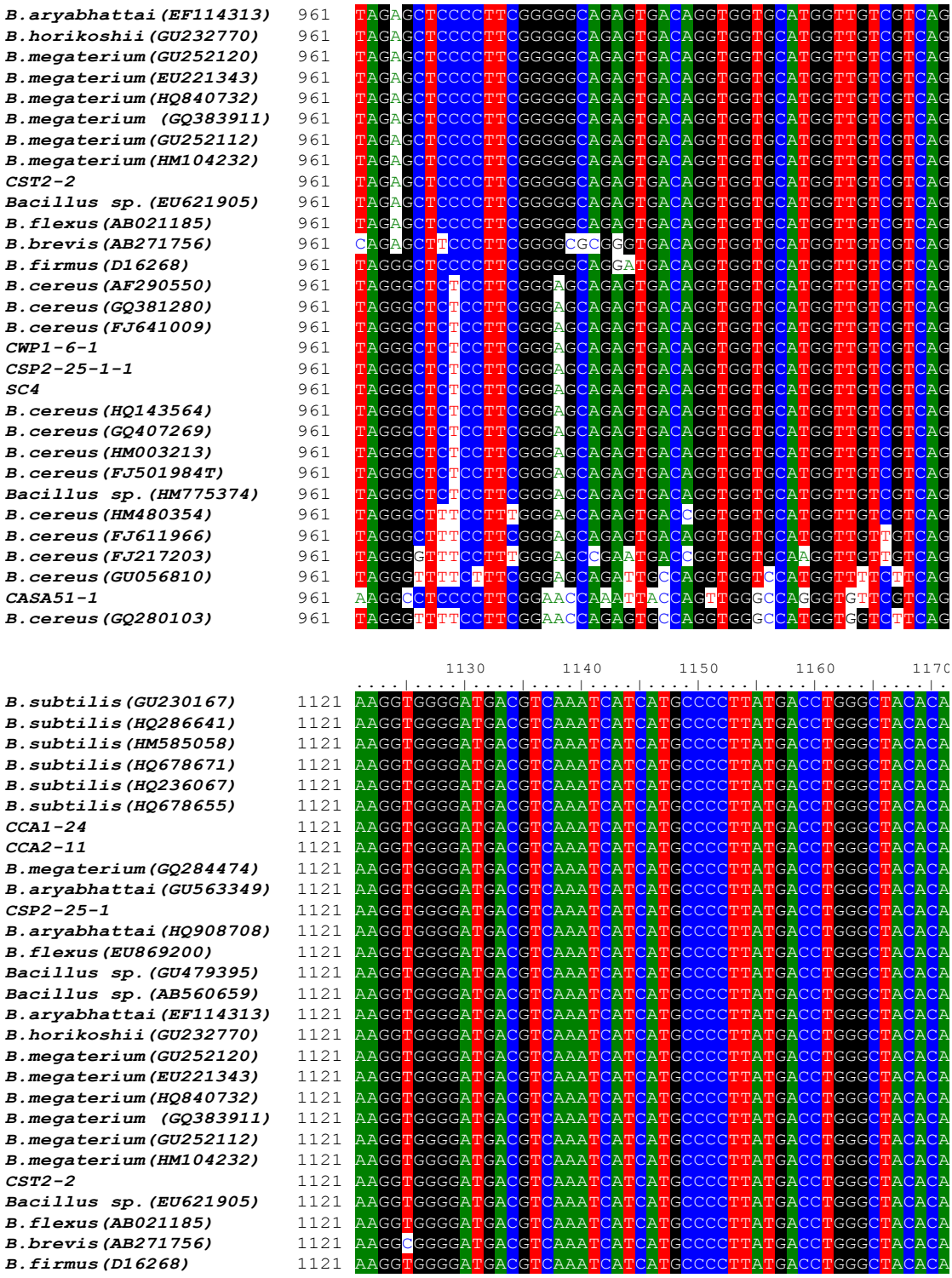

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

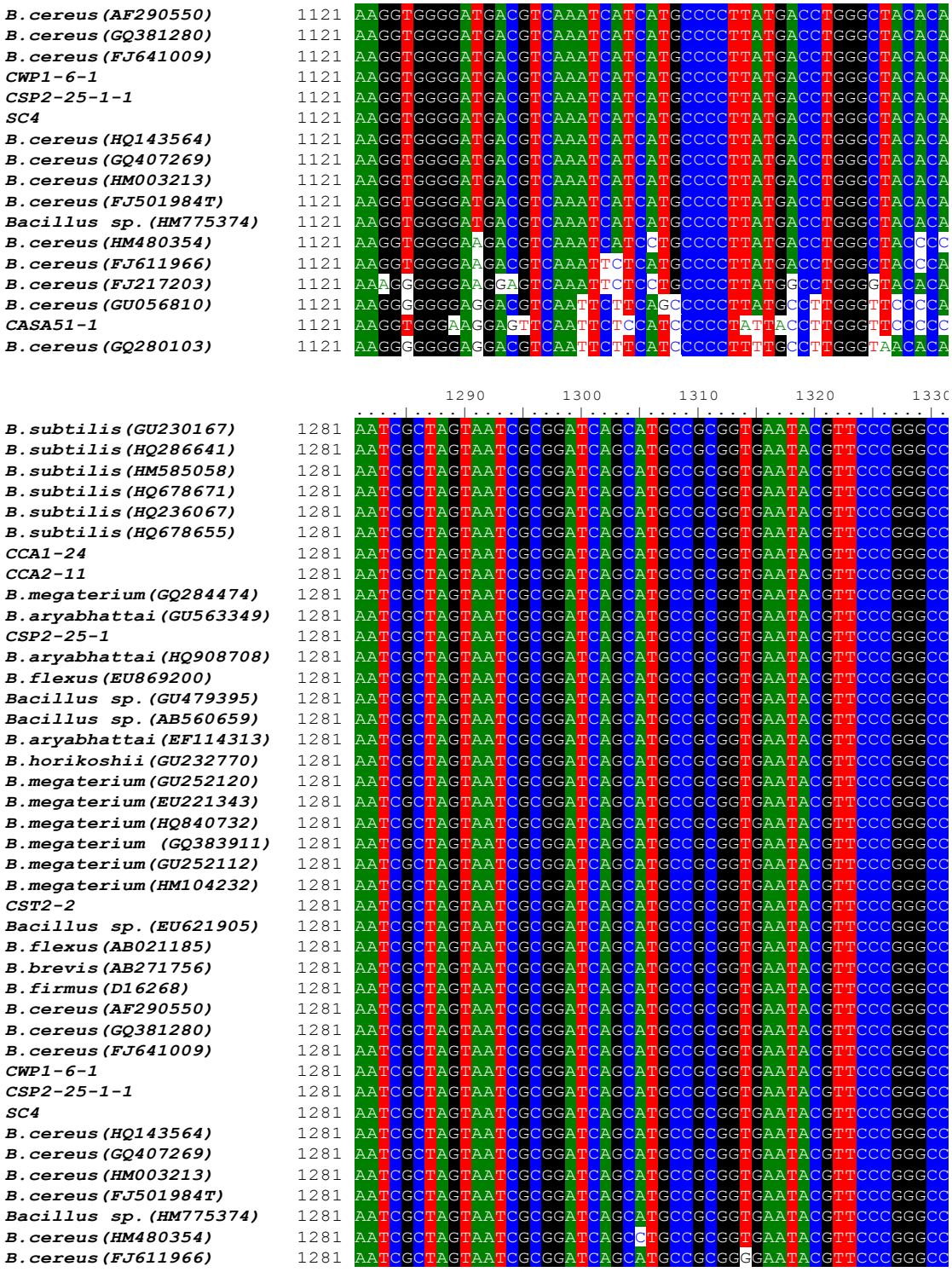

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

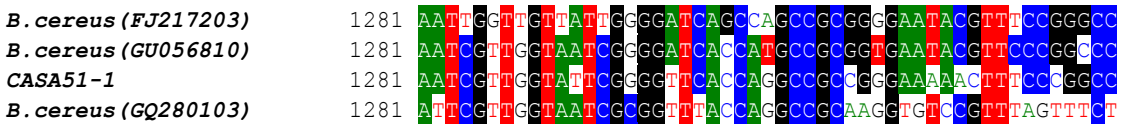

Figure 1.5C (Continued) Multiple sequence alignment of partial 16S rDNA using fD1 and rP2 primers of *Bacillus* sp. CCA2-11, CSP2-25-1, CST2-2, CCA1-24, CWP1-6-1, CSP2-25-1-1, SC4, and CASA51-1, examined compared to DNA sequences from GenBank database, by using Clustal X, BioEdit, and Mega 4 programs for phylogenetic tree construction.

APPENDIX D

LIST OF PRESENTATIONS

1. Poster Presentations

Chansatein, O. and Rodtong, S. (2009). **Development of cultivation media for screening of polyhydroxyalkanoates-producing bacteria isolated from cassava pulp**. The 2nd SUT Graduate Conference, January 21-22, 2009, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Chansatein, O. and Rodtong, S. (2009). **Nutrient elements require for PHAs accumulation of bacteria isolated from cassava pulp**. The Thai Society for Biotechnology, September 24-25, 2009, Queen Sirikit Nation Convention Centre, Bangkok, Thailand.

Chansatein, O. and Rodtong, S. (2009). **Monitoring of PHAs-producing bacteria using Nile red compared to PHAs accumulation**. The Thai Society for Biotechnology, September 24-25, 2009, Queen Sirikit Nation Convention Centre, Bangkok, Thailand.

Chansatein, O. and Rodtong, S. (2010). **Modification of Specimen Preparation Procedure for Detecting PHA Granule in Bacterial Cells Using Electron Microscopy**. The Thai Society for Biotechnology, October 20-22, 2010, Prince of Songkla University, Trang campus, Trang, Thailand.

Chansatein, O. and Rodtong, S. (2010). **Diversity Study of Polyhydroxyalkanoates-Producing Bacteria Isolated from Cassava Pulp Samples.** The Thai Society for Biotechnology, October 20-22, 2010, Prince of Songkla University, Trang campus, Trang, Thailand.

Development of cultivation media for screening of Polyhydroxyalkanoates producing bacteria isolated from cassava pulp

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Suranaree University of Technology, Nakhon Ratchasima, Thailand

Polyhydroxyalkanoates (PHAs) are biopolymers efficiently used as biodegradable plastics to replace environmentally unfriendly petroleum-derived plastics. The polymers can be synthesized by a wide range of microorganisms. Bacteria accumulate PHAs under conditions of nutrient stress particularly nitrogen or phosphorus limitations. PHAs-producing bacteria have been reported to be found in various environments. In this study, cultivation media were developed for screening a number of bacteria isolated from cassava pulp for their PHA production capability by modifying media described by potential references. Both complex and minimal media were developed for certain groups of isolated bacteria when cultured at 35 °C for 48 h. The isolates were tested for the presence of PHA accumulation by straining with Nile blue A and observed under the fluorescence microscope at excitation wave length of 650 nm. Alcaligenes eutrophus was used as the positive control of PHAs-producing strain. Twenty isolates were tested for PHA accumulation in their cells. It was found that the suitable complex medium composed of (per liter) 5 g yeast extract, 5 g polypeptone, 5 g tryptone, 2.5 g NaCl, and 10 g glucose; and minimal medium composed of 0.01 g $CaCl_2 \cdot 2H_2O$, 0.05 g ferrous ammonium citrate, 5 g glucose, 1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 g Na_2HPO_4, 2 g $(NH_4)_2SO_4$, 1 ml of trace element solution (0.2 g $CoCl_2 \cdot 6H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 5.56 g $FeSO_4 \cdot 7H_2O$, 0.3 g H_3BO_3, 0.03 g $MnCl_2 \cdot 4H_2O$, 0.03 g $NaMoO_4 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$). These media will be very useful for further screening of PHAs-producing bacteria isolated from cassava pulp.

Keywords:

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The 21st Annual Meeting and International Conference of Thai Society for Biotechnology
TSB 2009: "Biotechnology: A Solution to the Global Economic Crisis?"
24-25 September 2009: Queen Sirikit National Convention Center, Bangkok, Thailand

P-MF07

NUTRIENT ELEMENTS REQUIRE FOR PHAs ACCUMULATION OF BACTERIA ISOLATED FROM CASAVA PULP

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Polyhydroxyalkanoates (PHAs) are biopolymers that are efficiently used as biodegradable plastics to replace environmentally unfriendly petroleum-derived plastics. The polymers can be synthesized by a wide range of microorganisms. Bacteria accumulate PHAs under conditions of nutrient stress, particularly when a nitrogen or phosphate deficiency is present. In this study, major and minor elements in complex and minimal medium were investigated. Potassiumdihydrogenphosphate (KH_2PO_4) and disodiumhydrogenphosphate (Na_2HPO_4) in minimal medium requirement for PHAs accumulations of bacteria isolated from cassava pulp were determined. Both complex and minimal cultivation medium were developed by modifying media described by potential references. Ten formulas (A-J) of different KH_2PO_4 (0.45g, 0.83g, 0.1g, 1.0g, 1.4g and 1.5g) and Na_2HPO_4 (1.4g, 1.66g, 2.0g, 2.5g, and 3.0g) were applied. Different cell morphology rods and cocci which were isolated from cassava pulp code CSP1-4, CSP1-5, CWP2-16, CSC 2-2, N16, and CSP2-26 were used to develop cultivation of media testing, when cultured at 30 °C for 48 h. The isolates were tested for the presence of PHA accumulation by staining with Nile blue A. They were observed under a fluorescent microscope at excitation wave length of 650 nm. It was found that the suitable complex medium was composed of (per liter) 5 g yeast extract, 5 g polypeptone, 5 g tryptone, 2.5 g NaCl, and 10 g glucose; and minimal medium (B formula) composed of 0.01 g $CaCl_2 \cdot 2H_2O$, 0.05 g ferrous ammonium citrate, 10 g glucose, 1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 g Na_2HPO_4, 1 g $(NH_4)_2SO_4$, 1 ml of trace element solution (0.2 g $CoCl_2 \cdot 6H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 5.56 g $FeSO_4 \cdot 7H_2O$, 0.3 g H_3BO_3, 0.03 g $MnCl_2 \cdot 4H_2O$, 0.03 g $NaMoO_4 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$). Then, both suitable media were used for screening of PHAs-producing bacteria isolated from cassava pulp. Eighteen of 66 isolates can accumulate PHAs more than 50% of cell area when measure with Image-Pro Plus 6.0 program. Isolate code CASA40-1 can accumulate PHAs highest to 83% of cell area. Thus, use of appropriate nutrient elements for cultivation of PHAs-producing bacteria had an effect on PHAs accumulation in their cell.

Keywords: Polyhydroxyalkanoates (PHAs), PHAs-producing bacteria, PHAs accumulation, Elements

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P-MF08

MONITORING OF PHAs-PRODUCING BACTERIA USING NILE RED COMPARED TO PHAs ACCUMULATION

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Polyhydroxyalkanoates (PHAs) are biopolymers synthesized by a wide range of microorganisms, particularly bacteria. PHA granules in bacterial cells are detected based on cultural medium and straining with lipophilic dyes. In the present study, PHAs-producing bacteria isolated from cassava pulp were selected using Nile red dying technique and the relative accumulation of PHAs was monitored during cultivation. It was the first report for the detection of PHAs-producing bacteria within a few days. Three purified isolates code CSP1-5, CST2-2 and CST2-45-1 were cultured on complex, and then transferred to minimal cultivation medium which was supplemented with 0.5 mg/l Nile red (dissolved in 1 ml of dimethylsulfoxide). The minimal cultivation medium was separated to five sets each, for the first day to the fifth day. Petri dishes were incubated at 30 ^{o}C and detected for PHAs accumulation every day from the set one until the end of five. PHAs accumulation was tested by staining with Nile blue A, and then they were observed under a fluorescent microscope at excitation wave length of 650 nm. The result, slightly pink color on minimal medium was developed on the first day and more increased sequencing to dark pink on the last day. PHAs accumulation was observed when measured with Image-Pro Plus 6.0 program, the percentage of PHAs accumulation were 29.77, 38.64 and 23.72 of CSP1-5, CST2-2 and CST2-45-1 on the first day, respectively. There were the highest of PHAs accumulation occurred on the second day for isolate code CSP1-5 (44.25%), CST2-2 (62.73%) and the third day for CST2-45-1 (59.29%). The lipophilic dyes Nile red can be directly added to the medium to strain isolates that can produce PHAs. It could be applied to primary rapid screening for PHAs-producing bacteria.

Keywords: Nile red, Polyhydroxyalkanoates (PHAs), PHAs-producing bacteria, PHAs accumulation

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EB-P-22

Modification of specimen preparation procedure for detecting PHA granule in bacterial cells using electron microscopy

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Various microorganisms can produce the biopolymers, polyhydroxyalkanoates (PHA), in intracellular granular forms under nutrient-limited. The polymers serve as reserves of carbon and reducing equivalents to preserve cell survival during stress conditions. PHA granules in bacterial cells could be detected using specific cultural media and staining with lipophilic dyes such as Nile blue, Nile red, or Sudan black B. However, fluorescence straining may cause false-positive results or over explosions of PHA granules in the bacterial cells. Precise and consistent detection methodology for bacterial PHA granules is still desired. Electron microscopy is one of the perfect detection methods but there is no standard protocol available. In this study, specimen preparation procedure for efficient observation of bacterial PHA granules under transmission (TEM) and scanning electron microscopes (SEM) were focused. Four isolates of PHA-producing bacteria were selected for investigating. These isolates were cultured on modified complex and minimal media with incubating at 30°C for 48 h, then used for SEM and TEM specimen preparation. The fixative solution composing of 5% glutaraldehyde, 1% OsO_4 and 0.2M phosphate buffer was found to be suitable for this preparation. Acetone at series (20, 40, 60, 80, and 100%) gave better results than other organic solvent in the dehydration step. Epon viscosity embedding resin with incubating at 60°C for 24 h was sufficient for TEM polymerization. After sectioning, cupper grids were strained with uranyl acetate and lead acetate. This finding could enhance the detection of PHA granules using electron microscopy.

Keywords; Polyhydroxyalkanoates, PHA-producing bacteria, electron microscopy, SEM, TEM

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Prince of Songkla University, Trang Campus, Thailand, October 20-22, 2010

EB-P-23

Diversity study of polyhydroxyalkanoates-producing bacteria isolated from cassava pulp samples

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Polyhydroxyalkanoates (PHA) is a sort of biological polyester and function as carbon and energy reserves in bacterial cells. A wide range of bacterial species synthesize the polymers when a carbon source is provided in excess and essential growth nutrients were limited. In this study, the diversity of PHA-producing bacteria isolated from cassava pulp samples, was studied. The bacteria were classified into groups by comparing PHA accumulation levels in their cells and cell morphology. Seven hundred and seventy two bacterial isolates were detected for PHA accumulation using Nile blue A technique as well as monitoring by Nile red method. Eighty two isolates could accumulate PHA between 10 and 80% within their cell areas, which were observed under fluorescent microscope after cultivating on modified complex medium at 30°C for 48 h then transferred to modified minimal medium with incubating at the same condition and period. These PHA-producing bacteria were identified by their morphological and physiological characteristics. From cell morphology, the bacteria could be divided into 3 groups: Gram-positive cocci; endospore-forming Gram-positive rods and cocci; and regular, non-spore forming Gram-positive rods. From physiological characterization, 3 genera and 10 species were identified. These bacteria belonged to *Bacillus cereus*, *B. megaterium*, *B. stearothermophilus*, *B. firmus*, *B. mycoides*, *Staphylococcus cohnii*, *S. lentus*, *S. xylosus*, *S. sciuri*, and *Micrococcus* sp. Endospore-forming Gram-positive rods were found to accumulate PHA in higher content (70-80% of cell areas) than non-spore forming Gram-positive rods and cocci (10-60% of cell areas). These bacteria could have potential for further application in biopolymer production.

Keywords; Diversity, polyhydroxyalkanoates, PHA-producing bacteria, cassava pulp

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2005-2007	The Scholarship Under The Commission on Higher Education Staff Development Project for the Joint Ph.D. Program
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