Formation of specialized aerial architectures by *Rhodococcus* during utilization of vaporized *p*-cresol

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When grown with vaporized alkylphenols such as *p*-cresol as the sole carbon and energy source, several isolated *Rhodococcus* strains formed growth structures like miniature mushrooms, termed here specialized aerial architectures (SAA), that reached sizes of up to 0.8 mm in height. Microscopic examination allowed us to view the distinct developmental stages during the formation of SAA from a selected strain, *Rhodococcus* sp. KL96. Initially, mounds consisting of long rod cells arose from a lawn of cells, and then highly branched structures were formed from the mounds. During the secondary stage of development, branching began after long rod cells grew outward and twisted longitudinally, serving as growth points, and the cells at the base of the mound became short rods that supported upward growth. Cells in the highly fluffy structures were eventually converted, via reductive division, into structures that resembled cocci, with a diameter of approximately 0.5 µm, that were arranged in chains. Most cells inside the SAA underwent a phase variation in order to form wrinkled colonies from cells that originally formed smooth colonies. Approximately 2 months was needed for complete development of the SAA, and viable cells were recovered from SAA that were incubated for more than a year. An extracellular polymeric matrix layer and lipid bodies appeared to play an important role in structural integrity and as a metabolic energy source, respectively. To our knowledge, similar formation of aerial structures for the purpose of substrate utilization has not been reported previously for Gram-positive bacteria.

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INTRODUCTION

Bacteria often exhibit complex multicellular communities in response to environmental signal(s), allowing them to maintain their population at environmental extremes, which would not be feasible by single cells. For instance, *Pseudomonas aeruginosa* forms biofilms in the lungs of cystic fibrosis patients when the motility of the bacteria is restricted by host fluid (Matsui *et al.*, 2006), and myxobacteria and *Bacillus subtilis* form aerial structures

in response to nutrient depletion (Branda *et al.*, 2001; Dworkin & Kaiser, 1985). These organized multicellular structures enable the bacteria to exploit a wide variety of ecological niches by increasing their resource utilization, providing a defence against antagonists, providing protection from harsh environmental conditions, or all three (Brun & Shimkets, 2000). Microbial multicellular structure formation often requires collective and coordinated behaviour in which bacteria act as multicellular organisms (Shapiro, 1998). During recent years, the topic of multicellular cooperation among bacteria has been increasingly considered in the context of environmental applications as well as microbial pathogenesis.

The genus *Rhodococcus* is a phylogenetic group of non-spore-forming, aerobic, mycolate-containing Gram-positive bacteria that belong to the nocardioform actinomycetes (Bell *et al.*, 1998; Goodfellow *et al.*, 1998). They are widely spread throughout diverse environments, including

Abbreviations: EPM, extracellular polymeric matrix; SAA, specialized aerial architectures; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Rhodococcus* strains KL88, KL96 and KL97 are FJ555285, FJ555283 and FJ555284, respectively.

Four supplementary figures showing SAA formation by *Rhodococcus* and colony morphology are available with the online version of this paper.

soil and water; they are metabolically diverse and have the ability to degrade a wide range of xenobiotic and plant-derived molecules, playing a vital role in the turnover of organic matter in biogeochemical cycles (Goodfellow & Williams, 1983; Larkin *et al.*, 2005; Martinkova *et al.*, 2009; Warhurst & Fewson, 1994). These bacteria are well adapted to carbon starvation, chemical toxicity, adverse conditions and air-borne dispersal (Alvarez *et al.*, 2004; de Carvalho *et al.*, 2009; LeBlanc *et al.*, 2008; McLeod *et al.*, 2006; Sandhu *et al.*, 2009; Warhurst & Fewson, 1994). These bacteria are also known to have different cellular and growth patterns, showing cocci-shaped, rod-shaped and filamentous cells, with elementary branch patterns and microscopically visible hyphae (Goodfellow, 1989). However, unlike non-nocardioform actinomycetes such as *Streptomyces* the formation of fluffy colonies due to aerial hyphal filaments has not been reported for *Rhodococcus*.

During investigations of alkylphenol-degrading bacteria, we noticed the formation of highly fluffy aerial structures by *Rhodococcus* in response to alkylphenols such as *p*-cresol that were presented in the vapour phase. These three-dimensional structures were termed specialized aerial architectures (SAA). SAA were characterized by spatial and temporal developmental stages as if carried out by a bacterial multicellular community. In this study, we investigated the morphological changes during the formation of the aerial structures and the physiological characteristics of the cells in the SAA in order to further elucidate the functional role of SAA in the adaptation and survival of *Rhodococcus* in its environment.

METHODS

Strain isolation, identification, and culture conditions. Soil samples were collected from industrial areas located in Changwon, South Korea, and thoroughly suspended in 50 ml saline. After the debris settled, 20 µl of suspension was spread on minimal salts basal medium (MSB) (Stanier *et al.*, 1966) agar plates that contained 0.025 % (v/v) *p*-cresol, and the plates were incubated at 30 °C for 1 week. Pure colonies of *Rhodococcus* were isolated by repeated streaking on Luria–Bertani (LB) agar plates. Three strains were used for this study, labelled as KL88, KL96 and KL97, because these strains grew better than the other isolates. The partial 16S rDNA nucleotide sequences were determined as previously described (Kim *et al.*, 2004). The sequences generated were deposited in the NCBI nucleotide sequence database under the accession numbers FJ555285, FJ555283 and FJ555284 for strains KL88, KL96 and KL97, respectively. The 16S rDNA nucleotide sequences of the isolated strains were compared with the DNA sequences deposited in the NCBI database and the genus of the isolated strains was identified based on sequence homology. In addition, all strains were also identified based on MIDI-FAME profiling of the total cellular composite of fatty acids (Microbial ID, DE, USA).

Induction of aerial structures. In order to induce the aerial structures, single colonies from an LB agar plate were suspended to an OD₆₆₀ of 1.0 in saline and the cell suspension (30 µl) was spread on an MSB agar plate. A glass tube (length 5 cm, inner diameter 0.4 cm, outer diameter 0.6 cm) containing a small cotton plug was fixed

inside the lid of the Petri dish using Sellotape. Alkylphenols were added (50 µl) to the cotton plug. Then, plates were sealed with Parafilm and incubated in an inverted position for the required period at 25 °C unless otherwise indicated. Because the MSB agar plates were sealed with Parafilm, loss of water content from the MSB agar plate was less than 10 % for an incubation period of 10 months. A schematic representation of the setting is available in Lee & Veeranagouda (2009).

Photography and electron microscopy analyses. Photographs of the normal colonies were taken with a Digital Still Camera DSC-S75 with a zoom lens (Carl Zeiss). Aerial structures were photographed using an SMZ1500 stereomicroscope (Nikon) with an Infinity 1 camera (Lumenera) and a computer interface. Individual cells were examined using an Eclipse 50i phase-contrast microscope (Nikon). For the scanning electron microscopy (SEM) analysis, intact aerial structures were prepared and examined with a scanning electron microscope (Hitachi S4000 or JEOL JMS5610) as previously described (Lee & Veeranagouda, 2009). For the transmission electron microscopy (TEM) analysis, intact aerial structures were solidified using 1.5 % soft agar. These samples were processed as previously described and examined with a transmission electron microscope (JEOL JEM-1200EXII) (Choi *et al.*, 2007).

Measurement of dry weight, cell viability and metabolic activity. Dry weights of the aerial structures were determined by a gravimetric method. Aerial structures that formed beneath the glass tube were collected and dried at 100 °C for 10 h. For each measurement, three different settings were used. Viability of the cells present in intact 2- and 10-month-old SAA was determined by the BacLight LIVE/DEAD staining kit (L13152, Molecular Probes). Intact aerial structures were stained for 15 min with 4 µM SYTO 9 and 10 µM propidium iodide in 0.2 ml saline. These conditions were verified to distinguish the live and the 70 %-isopropyl-alcohol-treated dead cells when the experiment was carried out according to the manufacturer's protocol. The SAA were observed with a confocal laser scanning microscope (Zeiss LSM 510META) equipped with an AxioCam HRm camera for fluorescence imaging, as previously described (Lee & Veeranagouda, 2009). The metabolic activity of live cells was determined by measuring the level of cell respiration with tetrazolium blue chloride as an electron acceptor (Roslev & King, 1993), with modifications as previously described (Lee & Veeranagouda, 2009). By cellular respiration, the artificial electron acceptor is reduced to a water-insoluble red formazan. Formazan was dissolved in DMSO and measured at 546 nm. Metabolic activity measured from actively growing cells (15-day-old SAA) was considered as 100 % and residual metabolic activity was calculated at different time intervals.

Extraction and composition analysis of the extracellular polymeric matrix (EPM). EPM present in the aerial structures was isolated as previously described (Lee & Veeranagouda, 2009). The carbohydrate and DNA content of the EPM was determined by previously published methods (DuBois *et al.*, 1956; Paul & Myers, 1982). The protein content of the EPM was determined with a BCA kit (Pierce) using BSA as the protein standard. The sugar composition of the EPM was analysed by HPLC. Specifically, the dried EPM samples were treated with 2 M trifluoroacetic acid at 100 °C for 4 h at a concentration of 2 mg ml⁻¹. After the hydrolysates were freeze-dried, samples were dissolved in water and analysed by a High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection system (Dionex) using a CarboPac PA-1 column. The samples were eluted from the column with 18 mM NaOH for the monosaccharide and amino sugars, and were eluted with 0.1 M NaOH and 0.15 M sodium acetate for the sugar acids, at a flow rate of 1 ml min⁻¹. Commercial standards were used for the retention time standards.

RESULTS

Isolation of aerial-structure-forming *Rhodococcus*

Analysis of the amplified 16S rDNA nucleotide sequences and MIDI fatty acid profiles of the three isolated strains indicated that they belong to the genus *Rhodococcus*. The 16S rDNA nucleotide sequences of strains KL88 (KCTC 19690), KL96 (KCTC 19691) and KL97 (KCTC 19692) showed more than 99 % sequence identity with *Rhodococcus rhodochrous* (NCBI accession number AB183422), *R. opacus* (AY027586) and *R. jostii* (AB458522), respectively. All the isolated strains formed normal colonies on *p*-cresol-containing MSB agar plates (see Supplementary Fig. S1A1–A3, available with the online version of this paper). Interestingly, these strains formed unusual fluffy SAA on MSB agar plates when *p*-cresol vapour was supplied via a tube as described in Methods (Supplementary Fig. S1B1–B3 and C1–C3). SAA did not form in controls with glass tubes and cotton wool but no *p*-cresol. *Rhodococcus* sp. KL96 and KL97 colonies became slightly wrinkled when incubated for more than 1 week on LB agar. Wrinkled strains KL96 and KL97 formed SAA faster and across a wider area than the smooth strain KL88. None of these strains could develop SAA on *p*-cresol-containing MSB agar plates or LB agar plates when incubated for more than 3 months under the same incubation conditions. It is interesting to note that the SAA-forming ability has been conserved among different *Rhodococcus* species. Among the strains isolated, we focused our studies on *Rhodococcus* sp. KL96.

Factors affecting SAA formation

Rhodococcus sp. KL96 can utilize phenol, 3-ethylphenol, 4-ethylphenol, 4-butylphenol, 4-pentylphenol, *p*-cresol or *m*-cresol as the sole carbon and energy source, showing a similar range of growth substrates to '*Pseudomonas alkylphenolia*' KL28 (Jeong *et al.*, 2003). The best SAA formation was observed with vaporized *p*-cresol or *m*-cresol, whereas the formation of SAA was slow and not fully developed in the presence of vaporized phenol, 3-ethylphenol, 4-butylphenol or 4-pentylphenol (Supplementary Fig. S2). When the supply of vaporized *p*-cresol was stopped by removing the tube from the MSB agar plate at any time during the incubation period, no additional development was observed, indicating that these morphological changes require metabolic energy or a carbon source especially by vapour.

Morphogenesis phases of *Rhodococcus* SAA

In order to investigate the morphogenesis of SAA, the formation of SAA was continuously monitored under a stereomicroscope for 10 months. Initially, the *Rhodococcus* sp. KL96 cells that were spread on the MSB agar plates formed a uniform base within 4–6 days that was particularly predominant at the tube outlet (Fig. 1A). The cell mass away

Fig. 1. Morphogenesis of the SAA of *Rhodococcus* sp. KL96 as observed with a stereomicroscope or camera. Samples were prepared as described in the Methods and incubated at 25 °C for (A) 5 days, (B) 15 days, (C) 1 month, (D) 2 months or (E) 10 months. Photographs in A–D are top views. Bars: A–D, 0.1 mm; E, 0.2 mm.

from the tube was thinner. As time proceeded, small mounds projected out from the base that were probably due to local growth of the cell mass (Fig. 1B). These mounds gradually developed into spatially organized, smooth-surfaced, shiny bulbs. Then, small buds projected from the bulbs, especially from the bottom and sides of the bulb structure (Fig. 1C). Up to this stage, all the structures were shiny and watery. After 45–60 days, extensive branching of the bulb was observed, and after that period, the same shapes were maintained for more than a year (Fig. 1D, E). Extensive branching was first seen from SAA located away from the tube, which might have been due to limitations in the concentration of *p*-cresol. At this stage, the SAA were fluffy and easily detached from the MSB agar plates. For instance, the addition of water to the agar plates enabled one to float the SAA on water. The structures could not readily be broken into uniform suspensions in water, even with intense agitation, indicating either high hydrophobicity or that some extracellular material held the cells together. Typical sizes of the SAA were approximately 0.82 ± 0.10 mm $\times$ 0.32 ± 0.05 mm (height $\times$ width).

SEM observations of SAA

In order to obtain further information about cell size, position and their arrangements during SAA development, intact structures were fixed and examined by SEM. During the early stages of base formation (4–6 days), samples were too fragile to handle and process for SEM. A phase-contrast microscopic analysis revealed the predominant presence of long rods that were 3–4 μm in length. After base formation, long rod cells located on top of the mound were arranged in parallel with the agar surface, whereas cells located on the sides of the SAA were clubbed together and protruded out of the mound (Fig. 2A1–A3). With increased incubation times, bundles of cells present at the

centre of the mound twisted together and grew upwards, and this process prevailed primarily at places remote from the glass tube entrance, probably due to a limitation of *p*-cresol. In addition, these bundles were connected to each other by strings of cells that sometimes formed balls of cells (Fig. 2B1, B2). Cells at the tips of the SAA typically measured 6–7 μm in length, which possibly indicated active cellular growth (Fig. 2B3).

An intact 1-month-old SAA, which was located below the tube entrance, is shown in Fig. 2(C1). Bulbs were observed at the top of the structure, and there were numerous outgrowths from all sides of the SAA. Long rod cells with a branch at one end of the cell that formed a ‘Y’ shape were

Fig. 2. SEM photomicrographs showing the SAA of *Rhodococcus* sp. KL96 during morphogenesis. The SAA were fixed following incubation for 7 days (A1–A3), 15 days (B1–B3), 1 month (C1–C3) or 2 months (D1–D3). In A1, C1 and D1, the area within the black rectangle represents regions magnified in subsequent micrographs in the series. Bars: A1, C1 and D1, 0.1 mm; B1, 20 μm ; other images, 2 μm .

observed at the air interface to the bulb (Supplementary Fig. S3). Most cells were entangled with adjacent cells, and the cells at one end of the SAA were growing toward the *p*-cresol vapour, yielding cells with an orientation that was perpendicular to the surface of the bulb (Fig. 2C2). Perpendicular cell arrangements have been observed in other multicellular structures, including colonies and biofilms (Chang & Halverson, 2003; Enos-Berlage & McCarter, 2000; Lee & Veeranagouda, 2009). Closer examination of the images of the stalk of the SAA revealed that the short rod cells that were less than 1 µm in diameter had a more regularly compact arrangement (Fig. 2C3).

The 2-month-old SAA exhibited extensive branching of the SAA structures, especially on the top portion of the SAA (Fig. 2D1). Closer examination of the branches using SEM and phase-contrast microscopy revealed that the short rods formed by reductive division were arranged in chains (Fig. 2D2). These gross changes occurred following an incubation period of 45–60 days and may be due to nutrient depletion. In addition, short rod cells were maintained at the base and core regions of the 2-month-old SAA (Fig.

2D3). Without significant changes, SAA maintained their shape for more than a year. Cells present in 10-month-old SAA appeared to experience desiccation, because most cells at the air interface were shrunk.

TEM observations of SAA

Rhodococcus sp. KL96 cells from 2-month-old SAA were fixed and subjected to TEM analysis. TEM micrographs revealed short rod cells that measured 0.9–1.0 µm × 0.75 µm, as well as long rod cells undergoing reductive division (Fig. 3A). It was interesting to note that some of the long rod cells were undergoing irregular division, as shown in Fig. 3(B). In addition, the small rod cells were arranged in pairs, indicating further division, and yielded coccus cells (shown by arrows in Fig. 3A and Supplementary Fig. S4). Many cells exhibited spherical, electron-transparent inclusion bodies that sometimes contained a small, electron-dense inclusion body, as previously found for *Rhodococcus* sp. strain Q15 cells grown on diesel fuel (Whyte *et al.*, 1999). The transparent inclusion bodies have been shown to contain triacylglycer-

Fig. 3. TEM micrographs of cells in the SAA of *Rhodococcus* sp. KL96. (A) Rod cells from a 2-month-old SAA. Representative cells undergoing further division are indicated by arrows. A magnified image is also available in Supplementary Fig. S4. (B) Fragmentation pattern of long rod cells from a 2-month-old SAA. Cells undergoing irregular division are indicated within the grey oval. The spherical, electron-transparent inclusion bodies and one small, electron-dense inclusion body are indicated by thick and thin arrows, respectively. (C) Rod cells from a 7-month-old SAA. (D) Magnified image of a cell from a 7-month-old SAA. Bars: A, 2 µm; B, 0.2 µm; C, 1 µm; D, 0.1 µm.

ols and other neutral lipids. Members of the actinomycetes, particularly *Streptomyces* and *Rhodococcus*, reportedly accumulate lipid inclusion bodies during the stationary phase of growth (Alvarez & Steinbuchel, 2002). Bacteria have been shown to use these stored substances as a sole source of carbon and energy during prolonged starvation (Alvarez & Steinbuchel, 2002; Alvarez *et al.*, 2004). Inclusion bodies were also present in the 7-month-old SAA cells, and the rod cells measured $0.5\text{--}0.85\text{ }\mu\text{m} \times 0.45\text{ }\mu\text{m}$ in size (Fig. 3C and D), indicative of further reductive division of cells of the SAA during the prolonged incubation period when compared to the size of the cells shown in Fig. 3(A). The results were also confirmed by phase-contrast microscopic observations. The cell sizes are almost equivalent to those of ultramicrocells observed in *Pseudomonas* aerial structures (Lee & Veeranagouda, 2009).

Cell biomass, viability changes, and phase variation during SAA formation

Because development of SAA was associated with an accumulation of bacterial biomass, we measured, at various time intervals, the total size of the biomass formed under a glass tube providing *p*-cresol. The dry weight of the biomass obtained from each plate from the 15-day-old, 1-month-old, 2-month-old and 5-month-old SAA was 5.2 ± 0.1 , 11.3 ± 1.1 , 14.3 ± 1.2 and 13.0 ± 1.0 mg ($n=3$), respectively, indicating that growth of the SAA had mostly ceased after 2 months. The viability of cells present in SAA was measured using the BacLight LIVE/DEAD kit. As shown in Fig. 4(A), few dead cells were observed inside the 2-month-old SAA. Although the proportion of dead cells increased in the 10-month-old SAA, more than 50 % of the cells were still alive (Fig. 4B). By contrast, when a cell suspension from a 2-month-old SAA was dried by exposure to air for 6 h, more than 90 % of the cells were no longer viable. Thus, these results indicate that the SAA provides the cells with a shelter against starvation or desiccation.

When cells from 2-month-old SAA were spread onto LB agar, most of the colonies (>95 %) were a wrinkled,

uniform shape. Subsequent cultures of wrinkled colonies in LB medium did not convert to the original smooth colony type. However, the colonies from 15-day-old SAA samples were smooth, as originally isolated (Fig. 5A, B). When an identical number of these cells were initially inoculated for the formation of SAA, the wrinkled colony cells grew faster with closer spaced aggregates than the smooth colony cells (Fig. 5C, D). Furthermore, when both cell types were tested for the formation of pellicles at the surface of the LB liquid medium, the wrinkled colony cells formed a robust thick pellicle, but the smooth colony cells formed a thin and fragile pellicle after a 3 day incubation at 25 °C. These results indicate that the cells that formed wrinkled colonies have the selective or ecological advantage of proliferating in the aerial structures and pellicles.

Physiological characterization of SAA cells

The physiology of cells in the SAA was further examined by measuring the metabolic activity and EPM components. Changes in metabolic activity that occurred during the extended incubation period were measured as described in Methods. When compared with 15-day-old SAA, the metabolic activities of 2-, 5- and 10-month-old SAA were 47, 40 and 0 % ($\text{SD} < 5\%$, $n=3$), respectively. This result indicates that cells in the SAA gradually lost metabolic activity during the incubation period, and cells in the 10-month-old SAA had lost nearly all metabolic activity. Since ultramicrocells formed in aged SAA remain metabolically inactive but viable during prolonged starvation/desiccation conditions, we questioned whether these cells were also resistant to other forms of stress, such as high temperatures and oxidative stress. When cells from the 10-month-old SAA were exposed to 5 mM H_2O_2 for 10 min or 50 °C for 20 min, viable cells were recovered at the levels of 8.1 ± 3 and $8.3 \pm 4\%$ ($n=3$), respectively. The results indicated that the ultramicrocells may not possess physiological properties such as robust heat resistance, as shown by *Bacillus* spores (Brun & Shimkets, 2000). Because fresh vegetative cells of KL96 were mostly in chains, the experiments on the stress resistance could not be properly carried out with vegetative cells. In contrast, KL96 cells

Fig. 4. Live/dead staining of *Rhodococcus* sp. KL96 cells present in the SAA. In order to determine cell viability, (A) 2-month-old and (B) 10-month-old SAA were stained with the BacLight LIVE/DEAD kit and observed with a confocal laser scanning microscope. Live cells are indicated in green and dead cells are indicated in red. Bars, 10 μm .

Fig. 5. Phenotypic variations associated with the SAA cells. *Rhodococcus* sp. KL96 cells recovered from (A) 15-day-old and (B) 2-month-old SAA were spread on LB agar plates and incubated at 30 °C for 3 days. (C) SAA formed from the cells shown in (A). (D) SAA formed from the cells shown in (B). Plates were incubated at 30 °C for 1 month. Photographs are top views. Bars, 0.5 mm.

streaked on LB plates lost viability after 1 month incubation at 25 °C.

The EPM composition from 2-month-old SAA was determined as described in Methods. The carbohydrate, protein and DNA contents of the EPM were 423 ± 19 , 357 ± 18 and $78 \pm 3 \mu\text{g mg}^{-1}$ ($n=3$), respectively. These results are consistent with the fact that most of the microbial multicellular communities such as biofilms use EPM components, such as polysaccharides, proteins and DNA, to enhance their adhesion and to maintain the structures (Klausen *et al.*, 2006; Ma *et al.*, 2009; Sutherland, 2001). The HPLC analysis of the acid hydrolysates of the EPM showed that the polysaccharides consisted of L-fucose, L-rhamnose, D-glucosamine (possibly originally *N*-acetyl-D-glucosamine), D-galactose, D-glucose, D-mannose, D-xylose and D-glucuronic acid at a ratio of 1:1:1:10:4:2:1:2 ($\text{SD} < 10\%$). The same ratio of sugars was also detected from the EPM isolated from colonies from *p*-cresol-containing MSB agar, indicating that the major form of extracellular polysaccharide for aggregation in the two different niches is identical.

DISCUSSION

The results obtained in this investigation indicate that the isolated *Rhodococcus* strains readily form spatially organized multicellular structures (SAA) in the presence of alkylphenols, such as *p*-cresol, when presented in vapour form. Studies on the formation of SAA by strain *Rhodococcus* sp. KL96 revealed a series of distinct organizational phases that include substratum growth, mound formation, and outgrowth of groups of long cells from the mounds and bulb during highly branching stages. At early developmental phases, long twisted cells were laid in parallel to the agar surface (Fig. 2A2). This orientation

probably provides more surface area for cells to efficiently take up the *p*-cresol vapour and minerals from the agar surface. A similar arrangement of cells has been observed in the early stages of microcolony development by *Escherichia coli* and *Pseudomonas putida* (Chang & Halverson, 2003; Shapiro & Hsu, 1989). When the SAA were in actively growing stages, most long cells, including Y-shaped cells, were twisted in upright positions. These cellular forms may be due to apical growth as has been observed with other actinomycetes (Daniel & Errington, 2003), unequal division of cells as seen with TEM, or both (Fig. 3). The long cells present at the air interface of the bulb served as growth points (Fig. 2C2), whereas the mound of the short rod cells served as stalks of the SAA (Fig. 2C3). The long cells outgrown from the bulb became hyphae consisting of rod-type cells (Fig. 2D2). The highly branched structures of the SAA eventually made the SAA appear fluffy.

Aerial structures are preferred sites for the formation of spores, as can be seen in many bacteria, filamentous actinomycetes, slime moulds and fungi (Branda *et al.*, 2001; Brun & Shimkets, 2000; Flardh & Buttner, 2009). However, SAA of *Rhodococcus* sp. KL96 are a reservoir for ultramicrocells that are almost metabolically inactive. Although these ultramicrocells are not resistant to either H_2O_2 or heat treatment, they remain viable for an extended period of time (more than 1 year) under starvation/desiccation conditions. Hence, we looked for development/sporulation specific genes in the sequenced genome of *Rhodococcus jostii* RHA1, a bacterium closely related to strain KL96. Interestingly, several *Streptomyces* genes related to the formation of aerial mycelia, such as *bldC* (*R. jostii* RHA1 tag number ro02029) and *bldG* (ro03575), and genes related to sporulation/pigmentation, such as *whiA* (ro07176), *whiB* (ro03840, ro06193 and ro06345), *whiE* (ro00659), *whiH* (ro02364), *sigF1* (ro00098) and *sigF3* (ro04728) from *S. coelicolor* A3(2)

(Chater, 2001), were found to be conserved in the *R. jostii* RHA1 genome (McLeod *et al.*, 2006). The function of these rhodococcal genes remains to be discovered. In fact, SigF1 and SigF3 have been shown to play a key role in providing resistance against desiccation in *R. jostii* RHA1 (LeBlanc *et al.*, 2008).

Recently, we showed that the Gram-negative '*P. alkylphenolia*' KL28 strain can make specialized aerial structures under the same experimental conditions as shown in this study (Lee & Veeranagouda, 2009). In these two cases, the overall pathways for their development appear to be similar. First, mounds or domes form from the substratum and then these develop into highly branched forms in which the cells undergo reductive division(s) to form ultramicrocells. However, there are many developmental differences between the two strains. For instance, when making highly branched structures at the top of the mounds, serial bulb formation and continuous growth of the long rod cells are the main processes for '*P. alkylphenolia*' KL28 and *Rhodococcus* sp. KL96, respectively. In addition, at later developmental phases, the '*P. alkylphenolia*' KL28 cells are surrounded by thick EPM, whereas *Rhodococcus* sp. KL96 cells are surrounded by thin EPM or are directly exposed to the air. Results of the TEM examinations revealed that the structures of the former strain contained a massive number of lysed cells, but the structures of the latter strain did not. This finding may be due to differences between the two genera in the integrity of the cell wall or differences in the nutrient sources for metabolic activity required to maintain the dormant cells in the structures, such as autolysed materials or endogenous lipid bodies. In addition, differences in the overall shape of the SAA, size and timing of the branching exist during the formation of SAA by the two genera.

SAA formed by bacteria for utilization of vaporized alkylphenol will increase their surface area in three dimensions in order to become closer to the vaporized substrate. As Klausen *et al.* (2006) pointed out from studies on biofilms, SAA may be a specialized surface-associated microbial community that responds to the prevailing environmental conditions. In addition, the number of selective advantages of SAA formation are probably several-fold. SAA formation may also be a good strategy for breeding energy-minimized ultramicrocells and enabling their survival when the supply of nutrients runs out, the availability of water is limited, or both. SAA make colony transport to a new place by wind possible because of their lightness and hydrophobicity. SAA may open up new possibilities for utilizing bacteria in the treatment of contaminated air streams, and their production also signifies the importance of the mode of delivery of supplementary organic compounds for use with bacteria in bioremediation applications. Thus, SAA formed by bacteria represent an unusual example of multicellularity among the prokaryotes and therefore provide an opportunity to pursue questions about mineral transport to the SAA tips, cell-cell interactions, physical aspects of the

developmental process, and even the evolution of multicellularity. Currently, we are focusing on mutagenesis and proteomic studies in order to understand the role of genes differentially expressed during multicellular development in *Rhodococcus*.

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