Characterization of cyst cell formation in the purple photosynthetic bacterium *Rhodospirillum centenum*

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*Rhodospirillum centenum* is an anoxygenic photosynthetic bacterium that is capable of differentiating into several cell types. When grown phototrophically in liquid, cells exhibit a vibrioid shape and have a single polar flagellum. When grown on a solid surface, *R. centenum* will differentiate into rod-shaped swarm cells that display numerous lateral flagella. Upon starvation for nutrients, *R. centenum* also forms desiccation-resistant cysts. In this study, it was determined that *R. centenum* has heat- and desiccation-resistance properties similar to other cyst-forming species. In addition, microscopic analyses of the morphological changes that occur during cyst cell development were performed. It was observed that *R. centenum* typically forms multi-celled clusters of cysts that contain from four to more than 10 cells per cluster. It was also determined that cell density has a minor effect on the percentage of cyst cells formed, with cell densities of $10^5$–$10^7$ cells per 5 μl spot yielding the highest percentage of cyst cells. The striking similarities between the life cycle of *R. centenum* and the life cycle exhibited by *Azospirillum* spp. are discussed.

**INTRODUCTION**

Bacteria utilize several strategies for surviving environmental stresses, one of which is to form a metabolically dormant resting cell. By reducing the rate of metabolism, cells avoid starvation. In addition, the formation of complex cell structures, such as the cortex and coat of endospores, can protect a dormant cell against a variety of harmful environmental stimuli such as heat, desiccation and exposure to ultraviolet light (Nicholson et al., 2002; Takamatsu & Watabe, 2002). The endospore formed by Gram-positive bacteria, such as *Bacillus subtilis*, is the paradigm of prokaryotic resting cells, in both its dramatic resistance properties and in our understanding of endospore formation (Sonenshein, 2000; Stephenson & Hoch, 2002). However, other resting cell strategies exist, such as the cyst cells formed by *Azobacter vinelandii* (Sadoff, 1973). As with endospores, cyst cell development begins with a nutritional shift-down. In *Azobacter vinelandii*, the entire cell rounds up to form a large, spherical cell which is characterized by the presence of intracellular storage granules of poly-hydroxybutyrate (PHB) and a complex outer coat. The thick, multi-layered coat consists of an inner ‘intine’ layer composed of lipids and carbohydrates and an outer ‘exine’ layer composed of lipoproteins and lipopolysaccharides (Pope & Wyss, 1970). *Azobacter vinelandii* cysts are highly resistant to desiccation but, unlike endospores, they have minimal resistance to heat (Socolofsky & Wyss, 1962). Cyst formation has been reported in a wide range of proteobacteria, including the purple photosynthetic bacterium *Rhodospirillum centenum* (Favinger et al., 1989). Other cyst-forming species include members of the genera *Azospirillum* (Tarrand et al., 1978), *Methylobacter* (Whittenbury et al., 1970) and *Bdellovibrio* (Tudor & Conti, 1977). *Azospiillum brasilense* mutants that are impaired in cyst formation affect the efficiency of root colonization and nitrogenase activity (Katupitiya et al., 1995; Peregr Gerk et al., 2000). Furthermore, *Azospirillum* association with plant roots has been shown to stimulate crop growth, so a study of cyst formation may have practical agricultural applications (Okon & Itzigsohn, 1995; Steenhoudt & Vanderleyden, 2000). Even though cyst formation is a process that is integral to the biology of several diverse species, there is little known about the molecular mechanisms of cyst cell development.

*R. centenum* exhibits a complex life cycle consisting of three distinct cell types; swim cells, swarm cells and resting cysts (Favinger et al., 1989; Ragatz et al., 1995). When grown in liquid medium, the predominant cell type is the swim cell, which is vibrioid in shape and motile via a single polar flagellum. When grown on agar-solidified or viscous medium, the cells differentiate into rod-shaped swarm cells that express numerous lateral flagella. Entire colonies of swarm cells are capable of rapid movement across a solid surface (Jiang et al., 1998) similar to the swarming
behaviour of *Proteus mirabilis* (Bisset, 1973). In addition to swim and swarm cells, *R. centenum* also forms clusters of cyst cells when depleted of nutrients. The cysts formed by *R. centenum* are similar to those of *Azotobacter* (Sadoff, 1975) and *Azospirillum* spp. (Sadasivan & Neyra, 1985). Like those of *R. centenum*, certain *Azospirillum* spp. also exhibit swim and swarm cells (Moens et al., 1996), although *Azotobacter* spp. do not. Indeed, recent 16S rRNA gene phylogenetic analysis has determined that *R. centenum* forms a clade with *Azospirillum* spp. and thus may be considered a photosynthetic-capable *Azospirillum*-like organism (Stoffels et al., 2001). These similarities led us to examine more carefully the formation of *R. centenum* cyst cells, to determine how the physiology of the *R. centenum* resting cysts compares to the well-described physiology of *Azotobacter* cysts.

**METHODS**

**Bacterial strains and growth conditions.** Wild-type *R. centenum* strain ATCC 51521 was the sole bacterial strain used throughout this study. Culturing of *R. centenum* was performed at 37 °C under aerobic growth conditions in nutrient-rich CENS liquid medium. For rapid induction of cyst cells, agar-solidified CENBA medium and aerobic incubation at 42 °C were used. CENBA is a variation of the previously described CENMED minimal growth medium (Favinger et al., 1989) in which 20 mM butyrate substitutes for 20 mM pyruvate as the sole carbon source.

**Colony morphology.** For analysis of colony morphology, an overnight *R. centenum* culture was harvested and washed three times in phosphate buffer (40 mM KH₂PO₄/K₂HPO₄, pH 7·0). Serial dilutions appropriate to yielding isolated colonies were pipetted onto agar-solidified CENS medium and incubated at 42 °C. After 10 days growth of the original colonies, dilutions of fresh cultures were pipetted onto the same plates to allow direct comparison of mature and young colonies. Colonies were photographed using a Sony DSC-F707 digital camera.

**Phase-contrast microscopy.** For characterization of individual cells during the encystment process, *R. centenum* cultures were harvested and washed three times in phosphate buffer. Aliquots (5 µl) of cell suspensions were pipetted onto agar-solidified CENBA medium. For characterization of the cyst developmental cycle, cells were scraped from CENBA plates with wet mounts prepared at 12 h intervals over a 96 h period. Individual cells were viewed with a Nikon E800 light microscope equipped with a 100 × Plan Apo oil objective. Image capture was carried out with a Princeton Instruments cooled charge-coupled device (CCD) camera and METAMORPH imaging software, v.4.5.

**Electron microscopy.** *R. centenum* cultures were harvested and washed three times in phosphate buffer and then pipetted onto CENBA plates in 5 µl aliquots. After 1, 2 and 3 days incubation, the cell spots were harvested, fixed in 5% glutaraldehyde/100 mM HEPES/2 mM MgCl₂ and analysed by transmission electron microscopy as described previously (Favinger et al., 1989). Mature colonies of *R. centenum* were analysed by scanning electron microscopy, performed as described previously (Nickens et al., 1996).

**Analysis of cyst development, desiccation resistance and density dependence.** For analysis of the maturation of *R. centenum* cyst cells, wild-type *R. centenum* cultures were harvested and washed three times in phosphate buffer and 5 µl spots were pipetted onto CENBA plates. Spots were subsequently harvested daily for 6 days and resuspended in 1 ml phosphate buffer. Resuspended cells were sonicated for 5 s at low power (30% output, using a Microson ultrasonic cell disrupter) to disperse clumps [previous experiments by our group had shown that low-power sonication did not reduce the number of colony-forming units (c.f.u.) for vegetative cells but did effectively disperse clumps of cysts as evidenced by 10- to 100-fold increases in c.f.u. after sonication of cyst-induced cultures]. To quantitate the total number of viable cells (vegetative cells plus cyst cells), the resuspended cells were serially diluted onto CENS plates and incubated at 42 °C for 3 days. To quantitate the number of cyst cells, replicates of the total viable cell diluents were pipetted onto 0·45 µm filters, dried for 20 min at 22 °C, then desiccated at 42 °C for 3 days. Desiccated filters were then placed onto CENS plates for 2 days at 42 °C to allow outgrowth of surviving cells. Total colonies before and after desiccation were counted, with analysis repeated in triplicate.

For comparison of vegetative and cyst cell resistance to prolonged periods of desiccation, we analysed the desiccation resistance of both exponentially growing *R. centenum* cultures and cultures induced to form cysts. Cells were induced to form cysts by spotting washed cells of *R. centenum* onto CENBA medium as described above. Cyst-induced spots were harvested after 5 days incubation on CENBA and resuspended in 1 ml phosphate buffer. Resuspended cells were sonicated to disperse clumps, serially diluted in phosphate buffer and plated onto CENS plates to obtain a total viable cell count. Mid-exponential-phase cultures of exponentially growing *R. centenum* cells were obtained by growth in liquid CENS medium. Cells were harvested by centrifugation, washed three times in phosphate buffer and subjected to the same treatment as the cyst culture preparations. To obtain the number of desiccation-resistant cells, serial dilutions were also pipetted onto 0·45 µm filters as described above and desiccated for various lengths of time (1–14 days). Desiccated filters were placed onto CENS agar medium to allow outgrowth of surviving cells. After 2 days incubation at 42 °C, colony counts were obtained from dilutions which yielded defined colony growth. Analysis was performed in triplicate for each culture. Desiccation resistance was calculated by dividing the number of c.f.u. after desiccation by the number of c.f.u. prior to desiccation. Cell counts were then normalized to a total cell count of 5 × 10⁵ cells (actual total cell counts ranged from 1×10⁶ to 6×10⁶).

For analysis of density dependence on the formation of cysts, wild-type *R. centenum* vegetative cells were grown overnight in CENS medium, washed three times in phosphate buffer and concentrated to a final cell density of 2 × 10¹⁰ cells ml⁻¹. Serial dilutions were pipetted as 5 µl spots onto CENBA medium to yield inoculums of 1×10⁷–1×10⁹ cells per spot. After 5 days incubation at 42 °C, cell spots were harvested and resuspended in 1 ml phosphate buffer. Resuspensions were then sonicated to disperse clumps and serially diluted onto CENS plates and onto 0·45 µm filters to assay desiccation-resistance levels. Filters were desiccated then placed onto CENS plates as described above. The number of c.f.u. before and after desiccation was determined from three replicates of each dilution.

**Heat resistance.** Vegetative cells were harvested from a wild-type *R. centenum* culture grown at 37 °C in liquid CENS medium to an optical density at 650 nm of 0·2. Cells were washed three times and then resuspended in 1 ml phosphate buffer. Cyst-induced cultures were prepared by pipetting 5 µl spots of cells onto CENBA plates. The cells were then harvested after 5 days incubation at 42 °C and resuspended in 1 ml phosphate buffer. Cyst-induced and vegetative cell cultures were dispersed by brief sonication at low output power for 5 s with 100 µl aliquots incubated at 52 or 57 °C for 0, 15, 30 and 60 min. At each time point, surviving heat-treated vegetative and cysts cells were serially diluted in phosphate buffer and plated onto CENS medium to determine the number of surviving c.f.u.
RESULTS

Colony alteration during cyst development

Growth of *R. centenum* on agar-solidified CENS medium for a 3 day period leads to the production of red convex colonies (Fig. 1a, left). However, when colonies are incubated for longer durations (1–2 weeks), they develop a complex morphology composed of multiple tiers and striated ridges that protrude up and away from the agar surface (Fig. 1a, right). Significant changes in colony morphology have been observed for several developing bacteria, such as spore-forming *Bacillus* spp. (Branda et al., 2001) and cyst-forming *Azospirillum* spp. (Sadasivan & Neyra, 1987). None of these colony alterations are as dramatic as the elaborate fruiting bodies formed by some *Myxobacteria* spp. (Dworkin, 2000), which develop myxospores at aerial extensions of the fruiting-body structures. Alteration in colony morphology corresponds well with the appearance of cyst cells in *R. centenum*, but cysts are present throughout the entire colony as assayed by phase-contrast microscopy of dissected colony sections (data not shown). Scanning electron microscopy analysis of *R. centenum* cells from aged (1–2 week) colonies shows that vegetative and cyst cell types exist in a heterogeneous mixture throughout the colony (Fig. 1b), indicating that the process of cyst formation does not require elaborate cellular aggregation as does fruiting-body formation in *Myxobacteria*.

Wet mount microscopy, scanning electron microscopy (Fig. 1b) and thin-section transmission electron microscopy (Fig. 1c, d) all indicate that there are variable numbers of individual cellular units present in a single cyst ‘cluster’ as constituted by a well-defined intine–exine border. The number of cells per cyst cluster varies from approximately four cells that are observed soon after the colony enters cyst formation (Fig. 1c) to more than 10 cells per cyst cluster as the colony continues to age (Fig. 1d). Cysts comprising large clusters of cells, and cysts with multiple outer coats, have been observed in both *Azotobacter vinelandii* (Cagle & Vela, 1972) and *Azospirillum brasilense* (Sadasivan & Neyra, 1987). However, in these species, large cyst clusters are a rare occurrence with a ‘typical’ cyst containing only one cell. Another interesting aspect of the *R. centenum* cyst ultrastructure is the relatively thin outer coat.

![Fig. 1. Macroscopic and microscopic morphology of *R. centenum* cyst formation. (a) Typical colony morphology of *R. centenum* grown on nutrient-rich CENS medium. The shiny, convex colonies on the left are typical 3-day-old *R. centenum* colonies. The large, striated colony on the right is typical of a mature 2-week-old colony that is undergoing differentiation into cyst cells. (b) Scanning electron micrograph of *R. centenum* mature colony, showing a heterogeneous array of vibrioid-shaped vegetative cells and clusters of spherical cyst cells. (c) Thin-slice transmission electron micrograph depicting the ultrastructure of recently differentiated cyst cells, with three cells all surrounded by a continuous outer coat. The large electron-transparent granules in the cytosol of each cyst cell presumably consist of the polymer PHB. (d) Transmission electron micrograph depicting an older ‘giant’ cyst containing more than 10 cells surrounded by a common outer coat.](http://mic.sgmjournals.org)
vinelandii, the intine and exine layers can reach over 0.5 microns in thickness (Pope & Wyss, 1970), whereas in R. centenum cysts the exine is less than 0.1 microns in thickness, and the intine layer may only exist in the intercellular gaps present in each cyst cluster. There is also a reduction in the size of the intracellular PHB storage granules as the cysts progress from early to late cyst forms (Fig. 1c, d).

**Cellular maturation of cysts**

We also analysed the maturation of cysts by visually monitoring changes in cell morphology that occur after induction of cyst formation on butyrate-containing medium. To induce cyst development, we transferred cells to CENBA minimal medium, which contains 20 mM butyrate as the sole carbon source. Growth with butyrate derivatives as a sole carbon source has been shown to rapidly induce cyst formation in Azotobacter vinelandii (Stevenson & Socolofsky, 1966) and Azospirillum brasilense (Sadasivan & Neyra, 1985) in 2–3 days, as well as in R. centenum (Favinger et al., 1989). Wet mounts were then prepared at 12 h intervals over a 3 day period to observe morphological changes (Fig. 2a). At time zero, the culture consists of 100% vibrioid cells typical of vegetative swim cells. The first step in cyst formation becomes visible after 12 h, which is the appearance of intracellular granules. The light-refractile granules can be as large as the width of the cell and presumably consist of the polymer PHB, which has been shown to accumulate to up to 20% of the dry weight of cysts in R. centenum (Stadtwald-Demchick et al., 1990) as well as in cysts from other species (Stevenson & Socolofsky, 1973). After 24 h, the intracellular granules appear larger as cells take on an oblong shape. At this stage, motility becomes lethargic and by 36 h post-induction encysting cells lose motility concurrent with the accumulation of ejected flagella in the culture medium as observed by flagella staining (data not shown). From 36 to 48 h post-induction, cell division can be seen, but daughter cells no longer separate as they would in normal vegetative growth. Instead, cells divide but remain attached. At this time there is also an increase in the refractile aspect of the outer wall presumably due to the synthesis of the intine–exine outer coat. At 60 and 72 h, the outer coat becomes increasingly refractile, with subsequent cell divisions occurring that result in multi-celled cysts that are typical of R. centenum. Thin-slice transmission electron microscopy analysis (Fig. 2b, i–vi) also shows the ultrastructure of cells through these stages of cyst formation. Notable changes are the formation of large intracellular granules, alteration in cell shape and the formation of a thick outer coat during the development of cysts.

To better understand the process of cyst maturation, we assayed for the appearance of desiccation resistance after induction of cyst formation. For this analysis, we induced cyst formation in wild-type R. centenum by shifting to growth on butyrate-containing plates and then harvested cells every 24 h for 6 days of incubation on CENBA medium. After dispersal, the cells were serially diluted and then plated either onto CENS plates, to determine total viable cell count (vegetative plus cyst cells), or onto 0.45 µm filters. The filters were desiccated for 4 days at 42 °C prior to moving the filters onto CENS plates to allow outgrowth of surviving cells and determine the number of desiccation-resistant cells. Thus, a ratio of desiccation-resistant cells versus total viable cell counts gives an indication as to the rate of mature cyst development. The graph in Fig. 3 indicates that there are a negligible number of desiccation-resistant cells prior to the shift to butyrate-containing medium. However, after incubation on butyrate, there was a steady increase in the number of desiccation-resistant cells which plateau after 5 days. Thus, although mature cysts are microscopically observed within 72 h of induction, the maximum level of desiccation resistance is not achieved until 5 days after induction. This is probably due to a lack of synchronicity in the process of cyst formation, as the total number of mature cysts visible through microscopic analysis continues to increase over 3–5 days after induction. Indeed, Fig. 2(a) shows a field of aggregated multi-celled cysts commonly seen 120 h after induction, which

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**Fig. 2.** Stages of cyst cell development. (a) Phase-contrast microscopy depicting representative stages in R. centenum cyst formation. The panels labelled with white numbers show demonstrative cell types observed at 12 h time points after induction of cyst formation by a shift to butyrate-based CENBA medium. The panel with the black number depicts germinating cells 12 h after plating cyst cells onto rich CENS medium. (b) Thin-slice transmission electron micrographs portraying the ultrastructure of representative stages of cyst cell development in R. centenum. i And ii are from 1 day after cyst induction; iii and iv are from 2 days after cyst induction; v and vi are from 3 days after cyst induction.
corresponds with the maximum desiccation resistance observed in this organism.

Duration of desiccation resistance

*Azotobacter vinelandii* forms cysts that are resistant to desiccation for up to several months (Sadoff, 1975). To determine the extent of desiccation resistance in *R. centenum*, we subjected both vegetative and cyst cell cultures to periods of prolonged desiccation. Cultures were harvested and washed in phosphate buffer as before. Serial dilutions were pipetted directly onto 0.45 μm filters and placed in sterile Petri dishes where they were desiccated from 0 to 14 days prior to moving the filter onto CENS agar medium to allow outgrowth of surviving cells and assess the number of c.f.u. As shown in Fig. 4 (△), there is complete loss of viability of vegetative cells after just 1 day of desiccation. In the case of *R. centenum* cyst cultures (Fig. 4, ○), there was an initial decrease in viable cell counts to 33.8% of the total after 1 day of desiccation that we attribute to destruction of the vegetative cells that remained in the cyst-induced culture. This initial decline was followed by a more gradual decline over the next 14 days, with 3.2% of the cells surviving 2 weeks of desiccation. Extrapolation of the slope of the curve indicates that *R. centenum* cyst cells should survive desiccation for over 3 months.

Heat resistance

The endospores formed by Gram-positive bacteria typically exhibit resistance to high temperatures; however, cysts that are formed by Gram-negative species such as *Azotobacter* or *Azospirillum* typically have only moderate resistance to heat (Socolofsky & Wyss, 1962; Sadasivan & Neyra, 1987). To test the survivability of *R. centenum* cells to heat, we incubated vegetative and cyst cell cultures at 52 and 57°C for varying lengths of time. These assay temperatures are 5 and 10°C above the maximum vegetative growth temperature in this species, of 47°C (Stadtwald-Demchick et al., 1990). The survivability results shown in Fig. 5 indicate that there is only a slight loss in the viability of cyst-induced cultures when incubated at 52°C for as long as 30 min (81.8% survival) and that after 1 h incubation at this temperature there is still 65.5% survival. In contrast, incubation of vegetative cell cultures at 52°C leads to...
significant loss of viability, with only 0.0024% of cells surviving the first 30 min of incubation at 52 °C and only 0.000021% of cells surviving 1 h of incubation at this temperature. At 57 °C, both vegetative and cyst cells exhibit rapid loss of viability over a 30 min period. Thus, R. centenum cyst cells, like the cysts from other species, have only a modest resistance to elevated temperatures.

Involvement of cell density on cyst formation

Induction of endospores and myxospores has been shown to require a high cell density for development to proceed when a nutritional step-down occurs (Grossman & Losick, 1988; Kaplan & Plamann, 1996). We addressed whether cell density affects induction of cyst formation in R. centenum. For this assay, R. centenum cultures were harvested by centrifugation, washed and serially diluted. Serial dilutions of the R. centenum cell resuspensions were then pipetted onto butyrate-containing plates as 5 μl spots with initial inoculums ranging from 1 × 10¹ to 1 × 10⁸ cells per spot. Cells were incubated for 5 days to promote cyst formation. We then assayed for the percentage of cyst formation by resuspending the cell spots in 1 ml phosphate buffer and assaying for desiccation resistance (cyst cells) versus total viable cells (cyst cells plus vegetative cells). The result of this analysis (Fig. 6) shows that the percentage of cyst cell formation ranges from 18.7 to 51.9% of the total viable cell count and occurs maximally at initial cell densities of 10⁸–10⁹. Interestingly, there was no detectable minimum cell density requirement for cyst formation under these conditions, indicating that a cell density signal may not be essential for encystment to proceed on CENBA medium. The decrease in the percentage of cysts formed at high cell density (10⁸ cells per spot) is probably due to a lack of sufficient nutrient availability to enable efficient encystment.

Germination of cysts

Resting-cell formation is ultimately futile without subsequent regeneration of the vegetative cell. To analyse the process of germination in R. centenum, we desiccated cyst-induced cultures on 0.45 μm filters for 4 days, then shifted desiccated filters to a rich growth medium. In R. centenum, germination of cyst cells occurs much more rapidly than the formation of cyst cells. Within 6 h of plating onto rich growth medium, cyst cells can be observed through phase-contrast microscopy which show a decrease in the width and brightness of the highly refractile outer coat, presumably as a consequence of the degradation (or salvaging) of the outer coat. Individual cells within a cyst cluster appear smaller than the coat that surrounds them but there is neither movement nor cell division observed at this stage. After 12 h, there is an appearance of empty coats (husks) and ‘free’ cells which are predominantly oblong in shape.
Emerging cells are mostly pre-divisional cells and still contain large granules of PHB. After 18–24 h, return of the vegetative cell is observed with a recurrence of vibrioid and spiral shapes, an absence of intracellular granules and an active cell motility.

To better define the rate of germination, we resuspended desiccated cysts into liquid CENS medium and then followed germination by assaying for desiccation resistance from cells that were harvested at 12 h time points after induction of germination. By this assay, we observed that only 0-0001% of cells are desiccation-resistant 12 h after inducing germination. Twenty-four hours after induction, desiccation resistance has further decreased to the point where it is indistinguishable from vegetative cell cultures prior to cyst induction.

DISCUSSION

For a photosynthetic bacterium, *R. centenum* has an unusual life cycle (Fig. 7). It is the only photosynthetic organism for which swim cell and swarm cell differentiation has been observed (Nickens et al., 1996). Swarm cell differentiation allows rapid movement of *R. centenum* colonies across a solid surface, which could provide an advantage by allowing cells to migrate to optimal light conditions in a microbial mat (Ragatz et al., 1995). The formation of cysts is a developmental trait that has not been described for any other anoxygenic photosynthetic species. The ability to form desiccation- and heat-resistant cyst cells is presumably important for the survivability of *R. centenum* in the ecological niches that this organism is known to inhabit. Indeed, strains of *R. centenum* are typical isolates of hot springs and factory run-offs (Nickens et al., 1996). Successful enrichments of *R. centenum* have been reported using hot spring inoculums that were obtained from pools at temperatures as high as 58°C. This is despite the fact that *R. centenum* isolates obtained from such enrichments have a maximum vegetative growth temperature of 47°C. Presumably, the original enrichment inoculums contained cysts that allowed survivability at these elevated temperatures.

Phylogenetic trees based on 16S rRNA gene sequences have shown that *R. centenum* is closely related to species of the genus *Azospirillum* (Stoffels et al., 2001). This casts a new light on the developmental cycle observed with *R. centenum* since the physiological traits of *R. centenum* share a number of similarities with *Azospirillum* spp. *R. centenum* and *Azospirillum brasilense* each exhibit similar swim cells with distinct polar flagella, as well as swarm cells that exhibit surface motility through the production of distinctly different lateral flagella (Jiang et al., 1998; McClain et al., 2002; Moens et al., 1996; Scheludko et al., 1998). Likewise, *Azospirillum brasilense* also undergoes cyst formation as has been characterized in *R. centenum* (Sadasivan & Neyra, 1985; Sadasivan & Neyra, 1987; Stadtwald-Demchick et al., 1990).

Although there are several similarities between *R. centenum* and *Azospirillum* cysts, there is an important difference between these genera. For example, *Azospirillum* spp. typically contain only one cell per cyst coat. This is in contrast to the large cyst cell clusters that are typically formed by *R. centenum*. Indeed the number of cells per coat in *R. centenum* appears to increase as the cysts mature from an initial grouping of four cells per cyst to more than 10 cells per cyst coat as the cysts mature. One possibility is that the increasing number of cells is due to a delay in cyst-induced inhibition of chromosome replication in *R. centenum* leading to a large grouping of cells as the chromosomes subsequently segregate into daughter cyst cells. The appearance of multi-bodied cysts has been observed in both *Azotobacter vinelandii* and *Azospirillum brasilense*, but in these species it is a rare occurrence. Similar to myxobacteria, the formation of cyst clusters may be rationalized as a mechanism for ensuring a high cell density upon germination. The benefit for *R. centenum* could be the ability to quickly develop a cell density high enough to enable the colony-wide motility of swarm cells.

In addition to defining the physiology of *R. centenum* cyst cell development, we have begun performing a detailed genetic analysis of cyst development in this species. Mutations can be readily obtained that either significantly overproduce or fail to produce cysts. Characterization of these mutational events should shed light on the cyst developmental cycle in this, and in other, cyst-producing species.
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