Rodletless, a new Aspergillus developmental mutant induced by directed gene inactivation

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The Aspergillus nidulans CAN41 transcription unit is activated by the brlA regulatory gene early during development of the asexual reproductive apparatus, the conidiophore. Disruption of CAN41 results in a novel mutant phenotype in which conidiophore cells and spores lack an external wall layer, the rodlet layer, making them less hydrophobic than in the wild type and leading to inefficient spore dispersal. The rodletless mutation defines a new locus on chromosome III, rodA. rodA encodes a small, moderately hydrophobic polypeptide containing 8 cysteines arranged in a pattern similar to that observed in three hydrophobic cell wall proteins from the Holobasidiomycete Schizophyllum commune. We propose that the Aspergillus and Schizophyllum 8-cysteine polypeptides define a class of secreted, hydrophobic, fungal cell wall proteins that are important in the formation and function of aerial structures such as conidiophores and mushrooms.

[Key Words: Aspergillus nidulans; sporulation; spore wall; rodlet layer; reverse genetics]

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The production of asexual spores (conidia) by the filamentous fungus Aspergillus nidulans provides an example of programmed development that has been characterized by both genetic and molecular methods [for review, see Timberlake 1990]. Conidiation initiates in vegetative colonies following the achievement of developmental competence when environmental conditions are favorable (Axelrod et al. 1973) and is marked by the differentiation of specialized foot cells that give rise to aerial, multicellular, spore-bearing conidiophores (Oliver 1972; Mims et al. 1988). Martinelli and Clutterbuck (1971) estimated that 45–150 loci contribute specifically to spore production by comparing the frequency of developmental mutations with the frequency of selected, nondevelopmental mutations. Approximately 30 developmental mutants have been described in some detail [Clutterbuck 1987]. About one-third of these are defective in the initiation of sporulation, one-third produce abnormally pigmented or nonmaturing spores, and one-third are altered in conidiophore morphology.

In contrast to genetic estimates, molecular analyses suggest the involvement of a much larger number of genes. Approximately 1200 unique mRNAs accumulate preferentially during conidiation, ~200 of which occur in mature spores (Timberlake 1980; Zimmermann et al. 1980; Orr and Timberlake 1982). A large collection of cDNA and genomic clones corresponding to developmentally regulated transcripts has been made [Zimmermann et al. 1980; Boylan et al. 1987]. These clones have been characterized by the compilation of RNA accumulation profiles in wild-type and conidiation-defective mutants and in strains where developmental regulatory genes have been artificially activated in vegetative cells [Boylan et al. 1987; Adams et al. 1988; Mirabito et al. 1989; Timberlake 1990; Marshall and Timberlake 1991].

The disparity between the number of genes known to be specifically activated during conidiation and the number of developmental mutants leads to the question of what role, if any, conidiation-specific genes have in development. To begin addressing this question, we have chosen one cDNA clone, pCAN41, for detailed analysis: The transcript appears early during development, and its accumulation requires the early regulatory gene brlA but not the later regulatory genes abaA and wetA [Boylan et al. 1987; Adams et al. 1988; Mirabito et al. 1989; Marshall and Timberlake 1991]. In this study we have used DNA sequence comparisons and directed mutagenesis to determine the role in sporulation of the gene corresponding to pCAN41. Our results show that CAN41 identifies a new developmental locus that is essential for the formation of the outer hydrophobic wall layer of conidiophore cells and spores, the rodlet layer. We have named this gene rodA, for rodletless, and propose that it is a member of a class of genes encoding secreted fungal cell wall proteins that are important in the morphogenesis of hydrophobic, aerial hyphae and reproductive structures.
Results

Developmental regulation of CAN41

pCAN41 was selected in a screen for developmentally regulated A. nidulans cDNA clones (Boylan et al. 1987). The pattern of CAN41 transcript accumulation is shown in Figure 1. The transcript was present at low or undetectable levels in vegetative cells (hyphae) and mature conidia but at readily detectable levels in developmentally induced cultures, which contain hyphae, conidiophores, and conidia. These results imply that the CAN41 transcript accumulates preferentially in conidiophores. The CAN41 transcript failed to accumulate in a developmentally induced culture of a mutant strain lacking an active brlA early regulatory gene (see Timberlake 1990). In contrast, the transcript accumulated to apparently wild-type levels in developmentally induced cultures of mutant strains lacking the later-acting abaA and wetA regulatory genes. Thus, in the normal sequence of development, CAN41 expression is brlA dependent, but abaA and wetA independent. Finally, the CAN41 transcript accumulated in hyphae of a strain containing the brlA gene fused to the promoter from the alcohol dehydrogenase I gene alca [Adams et al. 1988] when the strain was grown on an inducing carbon source (threonine) but not when the strain was grown on a repressive carbon source [glucose]. Transcript accumulation under these conditions was also independent of the action of abaA and wetA and any regulatory genes acting upstream of brlA, demonstrating that brlA expression is sufficient for the induction of CAN41 transcript accumulation.

Transcriptional organization and sequence of CAN41

pCAN41 was used to identify a genomic clone, and the CAN41-coding region was localized to the 3.5-kb EcoRI-XhoI fragment shown in Figure 2A. The sequences of the cDNA and corresponding gene and flanking regions were determined and are given in Figure 2B. The CAN41 gene consists of three exons predicted to encode a 157-amino-acid residue, moderately hydrophobic [44% hydrophobic residues: A, F, I, L, M, P, and V] polypeptide. This polypeptide contains 8 cysteine residues, a single potential N-glycosylation site [Devereux et al. 1984], a highly hydrophobic amino-terminal sequence [see Fig. 8B, below], and has a net charge of -3 at pH 7. RodA has significant sequence similarity to three extracellular polypeptides from the wood-rotting fungus Schizophyllum commune [see Discussion, Schuren and Wessels 1990]. No other significant sequence similarities were identified in computer-assisted searches of the GenBank, EMBL, and NBRF data bases.

Inactivation of CAN41 leads to loss of the rodlet layer

To investigate the function of CAN41, we utilized the strategy outlined in Figure 3A to inactivate the gene [Timberlake and Marshall 1989]. Both haploid and diploid strains were transformed, as a heterozygous diploid would enable mutation recovery if CAN41 inactivation were recessively lethal. A screen of transformants by Southern blot analysis identified CAN41 deletion events among both haploids and diploids. Thus, CAN41 inactivation is not lethal. Transformants TMS015 [haploid] and TMS017 [diploid] were chosen for further study. Figure 3B shows that CAN41 had been replaced by the argB selective marker in the haploid. In the diploid, one copy of CAN41 had been replaced by the selective marker and one copy remained wild type.

Haploid CAN41 disruptants displayed a consistent mutant phenotype. As shown in Figure 4, A and B, after 2 days of growth on solid medium, a time when colonies were conidiating profusely, the centers of the mutant colonies became darker than the centers of wild-type colonies. The expression of the mutant phenotype did not affect nor was it affected by conidial pigmentation, as yellow-spored [yA2] or white-spored [wA3]. CAN41- double mutants produced conidia of the expected colors in dark-centered colonies [data not shown]. The CAN41...
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Figure 2. Transcriptional organization and sequence of the CAN41 locus. The pCAN41 cDNA clone was used to select a corresponding genomic clone, cosW16L5, from a cosmid library. An EcoRI-XhoI fragment encompassing the CAN41 transcription unit (A) was restriction mapped, and the region encoding CAN41 mRNA was sequenced on both strands, as was the cloned eDNA insert (B). Comparison of the eDNA and genomic sequences revealed the existence of two introns (underlined) containing consensus A. nidulans splice signals (boxed) (Ballance 1986). The transcription start site (arrow; +1) and the mRNA sequence upstream of the end of the cDNA insert (black dot) were determined by RNA sequence analysis with a primer corresponding to positions 375–392. The start site is preceded by two potential TATA boxes (overlined). The poly(A) addition site (*1 was deduced from the cDNA sequence. The cDNA/mRNA sequence possesses an open translation reading frame beginning with the 5'-proximal methionine codon, which is taken to specify the RodA polypeptide and is given in one-letter code below the DNA sequence. A single potential N-glycosylation site exists at residue 47 (circled) (Devereux et al. 1984). The GenBank accession number is M61113.
Figure 3. Inactivation of CAN41. (A) The CAN41 locus [1] includes two BglII fragments (map position 5.4–7.3 kb) corresponding to the 5' half of the CAN41 transcription unit and 1.5 kb of nontranscribed upstream sequence. These fragments were removed from a 9.5-kb BamHI–ClaI subclone and replaced with a BamHI fragment bearing the *A. nidulans argB* gene [2]. The linear BamHI–ClaI fragment of the resulting construct was used to transform haploid [RMS011] and diploid [RMS012] strains from which the *argB* locus had been deleted. The diagramed double crossover event produces the desired CAN41 mutation [3]. (B) Haploid and diploid transformants (20 of each) were screened by Southern blot analysis for the desired integration event. Four haploids and one diploid (RMS017) appeared to contain the desired deletion/substitution mutation. One haploid (TMS015) was selected for further study. DNA from the recipients [RMS011 (n) and RMS012 (2n)] and transformants [TMS015 (n) and TMS017 (2n)] was digested with BglII or BamHI and EcoRI, fractionated in a 1% agarose gel, transferred to a nylon membrane, and hybridized with the CAN41 9.5-kb BamHI–ClaI fragment. The smallest BglII restriction fragment from the wild-type locus is too small to be seen here. Sizes of bacteriophage λ HindIII fragments are given in kb.

type conidia resisted suspension in water, often forming dry layers on the outer surfaces of water droplets. Comparison of the wild-type and CAN41− mutant by transmission electron microscopy (TEM), shown in Figure 4, E and F, revealed that the mutant lacked the thin, proteinaceous, outer spore-wall layer, called the rodlet layer. Cole et al. [1979] observed an amorphous layer external to the rodlets in wild-type *Aspergillus niger* spores. However, we failed to detect such a layer in *A. nidulans* conidia [Fig. 4F and H]. The absence of the rodlet layer in the mutant was especially apparent in replicas of freeze-fractured specimens, as shown in Figure 4, G and H. The abundant, parallel rodlets were arranged in irregularly oriented bundles that covered the surfaces of wild-type conidia and of two conidiophore cell types, the primary [metulae] and secondary [phialides] sterigmata of the conidiophore [Fig. 4H]. These were absent in the mutant [Fig. 4G].
Figure 4. Phenotype of the CAN41 deletion strain. Photographs A, C, E, and G are of CAN41− strain TMS015, and B, D, F, and H are of CAN41+ strain RMS011. (A and B) Individual colonies of RMS011 and TMS015 photographed after 2 days of growth at 37°C. The CAN41− colony has a darkened central region (arrow) where water-soaked conidiophores have coalesced. (C and D) Free conidia suspended in water. CAN41+ conidia disperse readily, whereas mutant conidia adhere to one another, forming cylindrical spore masses. (E and F) Cross sections of outer wall layers of conidia viewed by TEM. Rodlets, the dark-staining, regular structures indicated by arrows on the CAN41+ conidium, are absent from the CAN41− conidium. (G and H) Replicas of freeze-fracture surfaces of conidia. Parallel bundles of rodlets are apparent in the CAN41+ conidium but absent from the mutant conidium.

Linkage of the CAN41 mutation and the rodletless phenotype

To determine whether the mutant phenotype described in the previous section and the CAN41 disruption were linked, we crossed TMS015 with RMS010 (Table 1). As demonstrated in Figure 5, both of these strains are deleted for the argB locus on chromosome III. TMS015 contains a single copy of argB+ integrated into the genome at the CAN41 locus. Thus, all arginine-independent progeny from this cross must contain a copy of argB integrated at CAN41, and all arginine-dependent progeny must contain an intact copy of CAN41. Of the 503 progeny scored, 257 were argB+ and had dark-centered colonies, and 246 were argB− and had wild-type colonies; no recombinants were detected. Thus, the introduced mutation and the mutant phenotype are separated by <0.5 cM.

CAN41 is a new gene: rodA

We refer to the phenotype of the CAN41 mutants as rodletless. To our knowledge, this phenotype had not been identified previously in A. nidulans. To confirm that the site-directed mutations identified a new locus, we mapped the mutation. We first established the chromosomal location of CAN41 by Southern blot analysis of CHEF-resolved chromosomes from the wild type and from a strain containing a reciprocal translocation between chromosomes V and VI (Käfer 1977; Brody and Carbon 1989). As shown in Figure 6, a CAN41-specific probe hybridized with the chromosome III + VI doublet in the wild type and with the chromosome III singlet in the translocation strain, permitting unambiguous assignment of the locus to chromosome III.

We crossed TMS015 with chromosome III mapping strain FGSC441 and detected linkage between rodletless and ActA and phenA, confirming the chromosome III assignment. The three separate crosses shown in Figure 7A were then used to map rodletless relative to the galA, carC, and ActA genes. As shown in Figure 7B, rodletless mapped between phenA and carC, a region containing no previously identified genes. We designated this new locus rodA.

Discussion

We generated a new A. nidulans developmental mutant by targeting disruption of a transcription unit, designated CAN41, that was chosen for investigation simply because it showed strong developmental regulation. CAN41 transcript begins to accumulate during development at about the time sterigmata appear and remains at high levels throughout the final stages of conidiophore formation and during spore differentiation and matura-
Table 1. A. nidulans strains

| Strain | Genotype | Source |
|--------|----------|--------|
| AJC472.1 | yA2, biA1, wetA6, veA1, trpC399 | A.J. Clutterbuck |
| FGSC4 | Glasgow wild type | Fungal Genetics Stock Center |
| FGSC26 | biA1; veA1 | Fungal Genetics Stock Center |
| FGSC40 | biA1; sA1; veA1 T1[V,VI] | Fungal Genetics Stock Center |
| FGSC288 | suA1ade20, yA2, ade20; wA3; galA1; pyroA4; facA303; sB3; nicB8; veA1, riboB2 | Fungal Genetics Stock Center |
| FGSC441 | riboA1, proA1, biA1; wA3; sC12; galA1. ActA1, phenA2, subB4pro, veA1 | Fungal Genetics Stock Center |
| IA015 | pabaA1, biA1; veA1, abaA14 | J. Aguirre |
| MH1179 | wA3; carC17; pyroA4; veA1 | M.J. Hynes |
| RMS010 | biA1; ΔargB::trpCΔB; methG1; veA1, trpC801 | M.A. Stringer |
| RMS011 | pabaA1, yA2; ΔargB::trpCΔB; veA1, trpC801 | M.A. Stringer |
| RMS012 | diploid: biA1; ΔargB::trpCΔB; veA1, trpC801 | this study |
| RMS023 | pabaA1, yA2; ΔargB::trpCΔB; veA1, trpC801 | this study |
| RMS024 | riboA1, pabaA1, yA2; ActA1, ΔrodA::argB; veA1b | this study |
| TMS015 | pabaA1, yA2; ΔargB::trpCΔB; ΔrodA::argB; veA1 trpC801 | this study |
| TMS017 | diploid: biA1; ΔargB::trpCΔB; methG1, veA1, trpC801 | this study |
| TTA021 | pabaA1, yA2; ΔargB::trpCΔB; ΔrodA::argB; veA1 trpC801 | Adams et al. [1988] |
| pabaA1, biA1; acaA[p]: brlA; abaA14, veA1 | Adams et al. [1988] |

aPartial genotype. May also contain galA1, trpC801, and proA1 and/or subB4pro.
bPartial genotype. May also contain galA1, ΔargB::trpCΔB, trpC801, and proA1 and/or subB4pro.

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scribed for A. nidulans prior to this study, and mapping of the mutant locus, here designated rodA, confirmed that this gene had not been identified previously.

Fairly large (>1 cm) colonies of rodA− mutants were easily distinguished from wild-type colonies due to the darkening of their centers where conidiophores had become water-soaked. However, smaller colonies were essentially indistinguishable from the wild type by direct visual examination. The failure of this aspect of the mutant phenotype to be expressed in small colonies probably explains why rodletless mutants were not identified in previous extensive screens for developmental mutants (Clutterbuck 1969, 1977). In these screens, colonies were plated at high densities under conditions that restricted colony growth to several millimeters, so the mutant phenotype would probably not have been apparent. The high density of colonies used in mutagenic screens can also lead to mutant remediation by "cross-feeding" of diffusible products produced by nearby nonmutants, as was observed for the Streptomyces mutant bld221 (Willey et al. 1991), which lacks a secreted peptide needed for aerial mycelium formation. However, cross-feeding is not likely to explain why rodA mutants were not identified previously, as rodA−/rodA− heterokaryons produced rodletless and normal conidiophores in close proximity to one another (M.A. Stringer and W.E. Timberlake, unpubl.). Our results and those from the study of the Streptomyces bld221 mutant reveal weaknesses in traditional screens for developmental mutants in micro-organisms. Not only are some mutants not easily identified in traditional screens, but some genes may not be susceptible to mutagenesis by classical techniques. For example, in Saccharomyces cerevisiae, extensive screens for temperature-sensitive lethal mutations after the use of several mutagens detected only six essential genes on chromosome I. Subsequent deletion analysis of six transcription units within a small segment of the same chromosome identified three new essential genes (Diehl and Pringle 1991; Harris and Pringle 1991). These studies may, in part, explain the discrepancy between estimates obtained from classical and molecular techniques for the number of genes involved in development. These results also suggest that reverse mutagenesis will add substantially to the number and types of developmental mutants, providing novel insights into the mechanisms controlling microbial morphogenesis.

The observation that rodA is required for formation of the rodlet layer of conidia, even though the rodA transcript does not accumulate in conidia, places this gene in a growing class of genes, including the conidial pigmentation genes yA (O’Hara and Timberlake 1989) and wA (Mayorga and Timberlake 1990), that begin to be transcribed in the sporeogenous phialides prior to sporulation, and whose products contribute directly to the formation of the specialized spore wall. Thus, the phialide, like the Bacillus spore mother cell (Losick et al. 1986), has a critical role in spor wall formation. We have observed that conidia produced in submerged culture (Adams et al. 1988) lack the rodlet layer and conidial laccase (T.C. Sewall, T.H. Adams, and W.E. Timberlake, unpubl.), the product of the yA gene, even though both yA and rodA transcripts accumulate (Adams et al. 1988). These results suggest that outer spore wall components are secreted onto the surface of the nascent spore by the...
Figure 5. Linkage of argB + to CAN41 −. DNA from a wild-type strain (FGSC4), the argB deletion, transformation recipient strain (RMS011), the CAN41 deletion strain (TMS015), and the argB deletion strain to which TMS015 was crossed (RMS010) was digested with BglII and subjected to Southern blot analysis. (A) The blot was hybridized with a DNA fragment containing the complete argB gene. (B) The blot in A was stripped and rehybridized with the CAN41 9.5-kb BamHI–ClaI DNA fragment (see Fig. 3). Sizes of bacteriophage λ HindIII fragments are given in kb.

Rodlet structures are a common feature of the aerial cells of filamentous bacteria and fungi (Cole et al. 1979). Beever and Dempsey (1978) reported that a rodletless (easily wettable) mutant of Neurospora crassa, an ascomycetous relative of A. nidulans, was deficient in the ability to disperse its conidia. The A. nidulans rodA mutant is also defective in spore dispersal, primarily due to adherence of the conidia to one another (see Fig. 4C) and liquid entrapment of conidia in the water-soaked centers of colonies. In A. nidulans, however, metulae and phialides, two nondispersed cell types that are not formed by Neurospora, normally possess rodlet layers that are absent in the mutant. It is possible that the rodlet layer on these cells contributes to spore dispersal by preventing water entrapment by the conidiophore or protects the cells from desiccation under conditions of low humidity, or both. We also observed rodlets on the surfaces of the conidiophore stalk and vesicle (T.C. Sewall and W.E. Timberlake, unpubl.). However, the stalk/vesicle rodlets were not arranged in a continuous layer but, instead, occurred singly, embedded in an amorphous matrix, and were not altered by rodA mutations. This result suggests that additional rodlet genes exist whose products are utilized in other aerial cell types.

Multiple genes encoding low-molecular-weight, hydrophobic, extracellular polypeptides, called hydrophobins, have been identified in the Holobasidiomycete S. commune. Schuren and Wessels (1990) described one gene, designated Sc3, that is expressed abundantly in both fruiting and nonfruiting cultures. Two related genes, Sc1 and Sc4, are expressed primarily during fruiting body development. The polypeptides encoded by these genes are secreted into the growth medium by submerged hyphae and accumulate in the cell walls of emerged hyphae. Wessels (1991) proposed that these proteins play a role in development of the aerial mycelium and formation of the fruiting bodies. One possibility is that these proteins diffuse away from submerged cells but polymerize in the walls of aerial cells, contributing to their hydrophobicity and structural rigidity. As shown in Figure 8A, there is significant sequence similarity between the Sc polypeptides and the predicted RodA polypeptide. Three shared features are particularly prominent. First, their amino termini resemble signal sequences, each with hydrophobic cores flanked by positively charged residues (Boyd and Beckwith 1990). This observation is in accord with the observed external

Figure 6. Assignment of CAN41 to chromosome III. A. nidulans chromosomes were isolated from FGSC4 and translocation strain FGSC40 [T1 VII, VII], fractionated by CHEF gel electrophoresis, and transferred to a nylon membrane. (A) The blot was hybridized with a mixture of radiolabeled FGSC26 DNA and a CAN41-specific DNA fragment. (B) It was then stripped and rehybridized with a CAN41-specific DNA fragment alone. Chromosomes are identified by Roman numerals.
Figure 7. Mapping CAN41 on chromosome III. [A] Linkage of CAN41 to chromosome III markers galA, carC, and ActA was tested in the crosses given in the first column. Progeny from all crosses were scored visually for the CAN41 phenotype. Additionally, progeny from the cross FGSC288 × TMS015 were tested for ability to utilize galactose as a sole carbon source; progeny from MH1179 × TMS015 were tested for carboxin resistance; and progeny from RMS023 × RMS024 were tested for resistance to cycloheximide. The number of parental and recombinant progeny for each cross is shown. [B] The position of CAN41 relative to galA, carC, and ActA is shown. Previously established map distances (uncorrected recombination percentages; Clutterbuck 1987) between relevant loci are given at top. (●) The centromere.

location of the Sc polypeptides and indicates that RodA is also secreted. Given that the Aspergillus conidial rodlet layer is composed largely of protein (Cole et al. 1979; Claverie-Martin et al. 1986), it is probable that RodA is a rodlet component. The second prominent feature of these polypeptides is conservation of internal hydrophobic domains, as shown for RodA and Sc4 in Figure 1168 GENES & DEVELOPMENT. Cysteine residues arranged in the same pattern, including the conserved tripeptide CCN. The conservation of these cysteines may be indicative of an important role in the functions of these secreted, hydrophobic polypeptides. This could be the cross-linking of the polypeptide chains. Site-specific mutations in the rodA gene and biochemical and cytochemical analyses of the RodA polypeptide should help to relate polypeptide structure to function.

Materials and methods
Aspergillus strains, growth conditions, and genetics
The genotypes of the A. nidulans strains used in this study are given in Table 1. RMS023 and RMS024 are meiotic progeny of TMS015 and FGSC441. Standard A. nidulans genetic techniques were used (Pontecorvo et al. 1953; Clutterbuck 1974). To score for the rodless mutation, strains were grown on agar-solubilized medium for 2 days at 37°C in a humid incubator. Haploidization of the diploid TMS017 was aided by streaking spores onto a benomyl concentration gradient generated by covering a sloped layer of fully supplemented solid medium containing 1.5 μg/ml of benomyl (a gift from Dupont) with an equal amount of medium without benomyl.

All strains were grown in appropriately supplemented minimal medium with NO3 as nitrogen source (Käfer 1977). For RNA isolations, wild-type and developmental mutant strains were grown as described previously (Timberlake 1980; Adams and Timberlake 1990). The vegetative culture of FGSC26 was harvested after 22 hr of submerged growth. For developmental RNA samples, strains were grown in submersed culture for 24 hr and harvested 25 hr after developmental induction. For in-
flanking and intervening residues were then aligned by using the GCG Gap program (Devereux et al. 1984). Amino acid identities are indicated by vertical bars, highly conserved substitutions by double dots, and less conserved substitutions by single dots between lines.

Hydrophobicity plots (B) of RodA and Sc4 were generated with the GCG Peptidestructure program (Devereux et al. 1984). The Kyte-Doolittle algorithm was used with a window size of 7. Points above the x-axis represent hydrophobic regions.

Figure 8. Comparison of the polypeptide sequences of rodA, Sc1, Sc3, and Sc4. In A, the inferred rodA polypeptide sequence and those of S. commune Sc1, Sc3, and Sc4 polypeptides (Schuren and Wessels 1990) were first aligned at the 8 cysteine residues (boxed). The flanking and intervening residues were then aligned by using the GCG Gap program (Devereux et al. 1984). Amino acid identities are indicated by vertical bars, highly conserved substitutions by double dots, and less conserved substitutions by single dots between lines.

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