Towards a reappraisal of the phenotype of the cell wall deficient *fz;sg;os-1* ("slime") triple mutant of *Neurospora crassa*

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Morphological mutants represent roughly 23% of seven hundred-odd distinct chromosomal loci of *N. crassa*, as listed by Perkins et al. (1982, Microbiol. Rev. 46:426). Probably the most radical phenotype among these strains is that of the *fz;sg;os-1* ("slime") triple mutant, which was isolated by Sterling Emerson (1963, Genetica 34:162) in a mutagenic experiment using an *os-1* strain. The "slime" strain has been systematically referred to in the literature as "a strain lacking cell wall and growing as protoplasts or plasmodium" (Perkins et al. 1982). Through the years, the fragile "slime" structures were frequently used as a source of organelles (Martinoia et al. 1979. Arch. Microbiol. 120:31), membranes (Scarborough, 1975. J. Biol. Chem. 250:1106) or for the study of membrane-bound enzymes (Brooks et al. 1983. J. Biol. Chem. 258:13909). "Slime" spheroplasts practically never revert to hyphal morphology; thus, the causes for impaired cell wall synthesis were investigated and attributed either to the lack of glucan synthase activity (Leal-Morales and Ruiz-Herrera, 1985. Exp. Mycol 9:28) or to improper ultrastructural characteristics of the organelles responsible for chitin synthesis: the chitosomes (Martinez et al. 1989. Biochem. Biophys. Acta 990:45).

"Slime" strains have been used in our laboratory for studying the effects of cell wall deficiency on the production of exported protein. Aside from an excess of protein secretion already reported by others (e.g. Bigger et al. 1972. J. Gen. Microbiol. 71:159), it was also observed that "slime" spheroplasts exhibit a pleiotropic defect for regulation of synthesis of several carbon-controlled catabolic exoenzymes including invertase; ß-glucosidase; alkaline protease; (Pietro et al. 1989. J. Gen. Microbiol. 135:1375); carboxy- methylcellulase; xylanase and pectinase (Polizeli et al. 1990. J. Gen. Microbiol. in press). This seems to be a general defect in the control of exoprotein production, because "slime" spheroplasts also secrete abundantly a cell wall-like particulate proteinaceous material, with self-assembling properties (Martinez et al. 1989. Arch. Microbiol. 125:25) and about fifty times more soluble protein than do wild-type strains. Moreover, the SDS-PAGE pattern of soluble extracellular protein secreted by "slime" is almost unaffected by the nutrient composition of the medium, in contrast to that of wild-type strains (unpublished).

Would these regulatory defects for protein secretion in "slime" be related to impaired cell well synthesis? This was a capital question bearing important implications concerning the role of the cell surface for processing environmental regulatory signals. This question was partially answered by reconsidering some of the details given in the original description of the "slime" strain (Emerson, 1963); specifically: that *fz;sg;os-1* ascospore segregants from crosses of "slime" and wild type ("slime-like") invariably develop into mycelium-forming cultures of abnormal morphology. These mycelial forms of "slime" (mycelial intermediate; Pietro et al. 1990. J. Gen. Microbiol. 136:121), when submitted to a protocol of filtration-enrichment selection (Nelson et al. 1975. Neurospora Newsl. 22:15), erratically produce clones with a permanent defect in cell wall biosynthesis, originating the well-known spheroplast-forming variant, stable "slime". A comparative study of mycelium-forming and spheroplast- forming derivatives of a single *fz;sg;os-1* segregant demonstrate that all defects in regulation of synthesis of exoenzymes arise concomitantly with the loss of cell wall. No new mutations seem to be selected by the procedure applied to mycelial forms for obtaining stable "slime" (Pietro et al. 1990). An analogous *N. crassa* system showing reversible formation and regeneration of stable spheroplasts was earlier reported by Selitrennikoff et al. (1981. Exp. Mycol. 5:155), using a temperature-sensitive osmotic mutant (*os-1*t, NM233t). Temperature shifts (25°C - 37°C) induce in the mutant the gain or loss, respectively, of cell wall forming activity. According to the authors, the mutant lost cell wall by incubation at 37°C.

Interestingly, the growth of "slime" spheroplasts is not restricted by several conditions which otherwise would inhibit growth of wall-forming strains, for instance the antibiotic Polyoxin B (a chitin synthase inhibitor, Selitrennikoff and Zucker, 1982. Exp. Mycol. 6:65); sorbose (a glucan synthase inhibitor; Quigley and Selitrennikoff, 1984. Exp. Mycol. 8:320); high osmotic concentrations (restrictive for growth of *os-1* mutants; Pietro et al. 1990) or temperature above 32°C (for "slimes" carrying the *cot-1* mutation; unpublished). Altogether these data stress the critical role of the cell surface for processing environmental information affecting cell growth.

In retrospect, one may realize that the real phenotype of the *fz;sg;os-1* multiple mutant strain has frequently been

confused with that of its wall-less form which is, after all, just the outcome of an obscure phenomenon of vegetative transformation. It may be possible that the immediate product of Emerson's mutagenic experiment was not the wall-less form, but a mycelium-forming strain, phenotypically similar to the mycelial "slime"-like ascospore segregants. These strains are characterized by a slow-growing, morphologically abnormal mycelium with parenchymatous surface growth, aerial hyphae practically absent and exhibiting a brown-yellowish (buff) pigmentation (Emerson, 1963). Newly isolated cultures are aconidial, but start to produce abundant microconidia- like structures after several cycles of transfer on agar medium of normal composition; otherwise the mycelial phenotype of "slime" is rather stable in standard medium (unpublished). The mycelium of "slime" is osmotically fragile and produces blobs of exudate on the surface of cultures made in solid medium, while cell wall ghosts always appear even in actively growing liquid cultures (Pietro et al. 1990). These strains are female-sterile, a characteristic attributed to the sg mutation (Emerson, 1963) but they are very effective as fertilizing parents. Ascospores carrying the *sg* mutation germinate spontaneously, not requiring heat activation; however, if submitted to a heat treatment (45 min at 60°C) these ascospores do lose viability (unpublished). This fact suggests that the thermotolerance inherent in the ascospore is independent of the mechanism which blocks germination unless thermal or chemical stress is imposed. On cultivation in liquid medium of high osmolarity (e.g. 1.0 M sorbitol) and in the presence of 1% sorbose, mycelial forms of "slime" tend to release spheroplasts; nonetheless these spheroplasts generally revert to mycelial forms in media of normal osmolarity. Occasionally, clones with a weakened ability for synthesizing cell wall are produced (spheroplast/hyphal intermediate; Pietro et al. 1990). These forms grow as a pure spheroplast population even in media of moderate osmolarity (0.2 M sorbitol), but produce hyphae under normal culture conditions. After a few more transfers under selective conditions, spheroplast/hyphal intermediates may eventually produce stable "slime" cultures.

Apparently, during continuous growth under selective pressure fz;sg;os-1 mutants suffer a progressive and irreversible loss of cell wall synthesizing ability, reminiscent of a phenomenon of epigenetic transformation, because no new mutation can be demonstrated. Together with the loss of cell wall, other important mechanisms for recognition of environmental signals are lost as well (Selitrennikoff, 1979. Exp. Mycol. 3:363). A possible explanation for the loss of cell wall is that in *fz;sg;os-1* mutants cell growth and cell wall biosynthesis are uncoupled, the latter being too limited to encompass the growth of cells proliferating at a fast rate. This phenomenon may be observed at early stages of ascospore germination or at the border of older colonies growing in solid medium. This effect can be dramatically exaggerated when cell wall synthesis is affected by the use of inhibitory substances such as sorbose. The loss of cell wall-synthesizing ability is gradual and irreversible, suggesting the existence of discrete units which might act as "focuses" or "primers" for cell wall synthesis, and that might be somewhat independent of immediate gene expression. These hypothetical units may be progressively lost during vegetative propagation under selective pressure, eventually giving rise to stable "slime" clones.

If the mechanism by which stable "slime" clones arise from strains with cell wall forming ability is a fascinating subject for investigation, no less intriguing is the process by which *fz;sg;os-1* ascospore segregants from crosses of spheroplasts and wild type reacquire the ability to synthesize cell wall. It seems unlikely that this phenomenon is produced by genome rearrangements. On the other hand, the female sterility of "slime" precludes genetic analysis for non-chromosomal inheritance. In this context it is worth noting that stable "slimes" cannot be recovered from "slime"/wild type heterokaryons just by conidial plating, rather it is necessary to use the time consuming method of filtration- enrichment selection. Preliminary evidence in our laboratory suggests that *fz;sg;os-1* homokaryons newly resolved from "slime"/wildtype heterokaryons may in fact be mycelial strains (unpublished).

The study of mycelial phenotypes of the "slime" strain may open new and exciting prospects for a better understanding of cell wall inheritance and morphogenesis, and of the role of the cell surface as a primary site for recognition of environmental stimuli. Other interesting aspects of the mycelial-spheroplast transformation of "slime" strains are the changes in structure and composition of the cell wall which must occur during this process. Since it is obvious that, in principle, *fz;sg;os-1* mutant strains are capable of cell wall synthesis, the causes for the reported lack of activity of polysaccharide synthesizing enzymes in "slime" spheroplasts needs reconsideration. Moreover, since it is unknown whether defective cell wall of "slime" is a result of reduced biosynthetic ability or excessive degradation, the activity of wall lytic enzymes may also deserve to be considered.