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The Effect of the Antifungal Secretions of a Novel Pseudomonas Species on Cell Hydrophobicity, Growth Viability and Ion Permeability of *Candida albicans*

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ABSTRACT

A novel *Pseudomonas* species, designated as *P. uticensis*, was isolated from the cutaneous flora of red-backed salamanders due to its ability to produce antifungal secretions. We hypothesize that at least one antifungal secretion produced by *P. uticensis* is a Ca^{2+} ionophore, because exposure of *Neurospora crassa* to bacterial secretions results in abnormal hyphal branching patterns. *Candida albicans* is a polymorphic fungus that can grow as a budding yeast, as pseudohyphae, or as true hyphae. Its ability to shift between the yeast-to-hyphal state is essential for its virulence. Our previous research showed that the antifungal secretions of *P. uticensis* inhibit the yeast-to-hyphal transition in *C. albicans*, as well as biofilm development. Inhibition was more pronounced in hyphal cultures induced by minimal media with 5% newborn calf serum (NCS), compared to those induced by 10 mM N-acetylglucosamine (NAG). The purpose of this research was to determine if the antifungal secretions from *P. uticensis* effect cell hydrophobicity, growth and viability in *C. albicans*, as well as ion permeability. A sterile supernatant was prepared from TSYE broth cultures of *P. uticensis* and was added in increasing concentrations to *C. albicans*, in media with either NCS or NAG. Both media were selected to induce the hyphal state, and the hydrophobicity index was determined. Both NCS and NAG cultures showed at least a 50% decrease in cell hydrophobicity as increasing concentrations of *P. uticensis* supernatant were added, indicating the inhibition of hyphal formation. Furthermore, a dose-dependent inhibition of *C. albicans* growth was observed with increasing concentrations of supernatant, with a biomass reduction up to 60%. A 50% decline in cell viability was apparent with 10% concentration of supernatant; no further decline was observed with higher concentrations, suggesting a threshold effect. At least one of the *P. uticensis* antifungal secretions is a Ca^{2+} ionophore, as a direct correlation between increased supernatant concentrations and change in cytoplasmic Ca^{2+} levels was observed. We suspect that this effect is Ca^{2+} specific because no change in cytoplasmic pH was detected under the same culture conditions. Studies to identify the Ca^{2+} ionophore involved in disruption of Ca^{2+} homeostasis in *C. albicans* morphogenesis are in progress.

BACKGROUND

Candida albicans is a polymorphic fungus that is found in the human digestive tract as well as on the skin. It can grow as an ovoid budding yeast, as elongated ellipsoid cells that are still attached at a constricted separation site, also known as pseudohyphae, or as true hyphae. Pseudophyphae differ from hyphae in that there are constrictions between the neck of the mother cell and the bud of every following connection with inconsistent width measurements (Berman *et al.*, 2004). *C. albicans* is known to be an opportunistic pathogen, and previous research has found that dimorphism is essential for its pathogenicity (Jacobsen *et al.*, 2012).

Previous studies have demonstrated genes and signaling pathways that are involved in the yeast-to-hyphal transition. Specifically, Efg1 is identified as a basic helix-loop-helix protein that plays a major role in the growth and formation of hyphal morphogenesis. Additionally, Tup1 is also an important transcriptional co-repressor and is responsible for regulating the yeast-to-hyphae transition (Haoping *et al.*, 2001). Other important factors can also be attributed to the morphology of *C. albicans*, such as pH, temperature, and quorum sensing (Hube *et al.*, 2013).

It is known that when *C. ablicans* switches from the yeast to hyphal form, infection and invasion of the host tissue increases. Certain compounds, such as azole drugs, have been known to act as antifungal agents, and it has been shown that azole drugs inhibit the transition from yeast to hyphae in *C. albicans* (Ha *et al.*, 1999). Work in our lab has also demonstrated that the antimicrobial lipid sphinganine also inhibits the yeast-to-hyphal transition in *C. albicans* (Gerlach et al., 1996)

A novel *Pseudomonas* species, designated as UC17F4 was previously isolated in our laboratory from the microbial flora of female red-backed salamanders, and exhibits potent antifungal activity (van Kessel et al, 2003). Another interesting feature of UC17F4 is the production of the brown pigment pyomelanin. Pyomelanin production in UC17F4 is light-dependent (Kracke et al, 2011; Benzing *et al.*,2012), as well as cell density-dependent (Seifert *et al.*, 2013).
While UC17F4 was originally isolated due to its ability to produce powerful antifungal compounds, we know relatively little about the mechanism of its antifungal activity. In thee present study, we investigate the inhibitory effects of the antifungal properties of UC17F4 on the morphogenic transition in *C. albicans*.

METHODS

Preparation of bacterial supernatants: Overnight cultures of *P. uticensis* were prepared in tryptic soy-yeast extract (TSYE) broth. Cutures were centrifuged at 8,000xG for 20 minutes, and supernatants were collected and subjected to sterile filtration through 0.45 μm filters, and stored at -20ºC.

Hyphal induction studies: *C. albicans* 3153A yeast-phase cells were inoculated at 10^6 cells/ml into one of two hyphal inducing medias: Vogel's Minimal Media (Vogel, 1956) with 5% newborn calf serum (NCS) or 1% Tryptone with 10 mM N-acetylglucosamine (NAG). Treatment groups were prepared with increasing concentrations of UC17F4 supernatant, ranging from 0 to 50%. Cultures were then incubated for 4 hours in a 37°C water bath, shaking at 250 rpm.

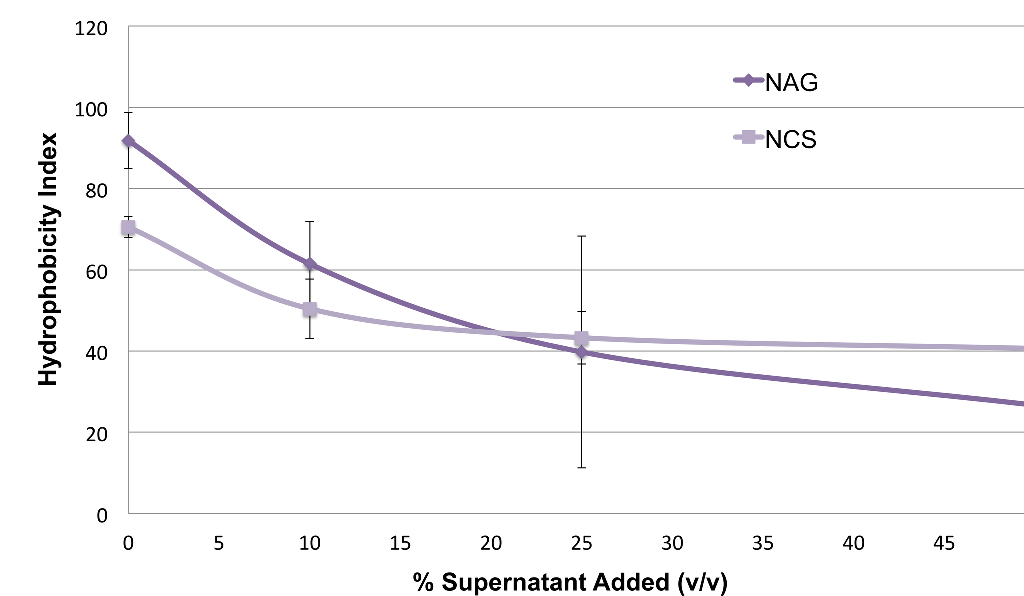

Fig. 1. Supernatant from *P. uticensis* effects cell hydrophobicity of *Candida albicans*. Yeast cells were inoculated at 10^7 cells/ml with increasing concentrations of *P. uticensis* supernatant. Cells were incubated at 37°C for 1 hr. Cells were pelleted and resuspended in PBS. The optical density was read at 660nm, followed by the addition of 400 μl of xylene. The turbidity of the aqueous layer was measured at 660nm, then the hydrophobicity index was computed. Each point represents the average Hydrophobic Index at 660nm. As concentration of supernatant increase, cell hydrophobicity decreases, representing a decline in hyphal cell presence.

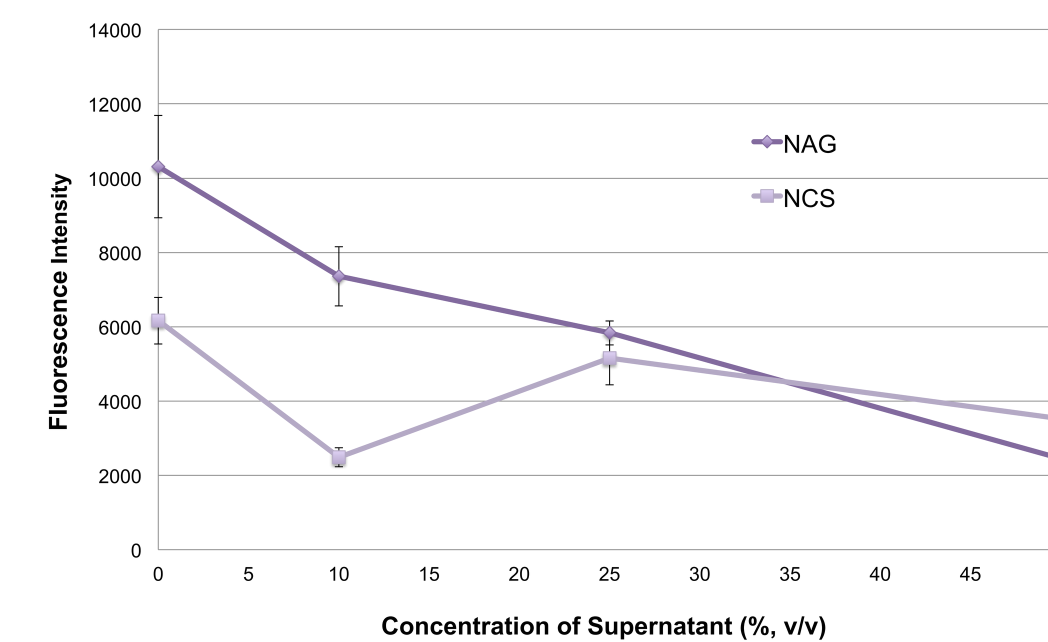

Fig. 2. Supernatant from *P. uticensis* effects cell growth of *Candida albicans*. Yeast cells were inoculated at 10^6 cells/ml with increasing concentrations of *P. uticensis* supernatant. Cells were incubated at 37°C for 3 hr. Post-incubation, cells were stained with 100 μl of diluted Calcofluor White, and then incubated for an additional 45 mins at 30°C in the dark. Cells were centrifuged and resuspended in TE buffer. Biomass was determined using a fluorescent microplate reader. Each point represents the average of 3 determinations.

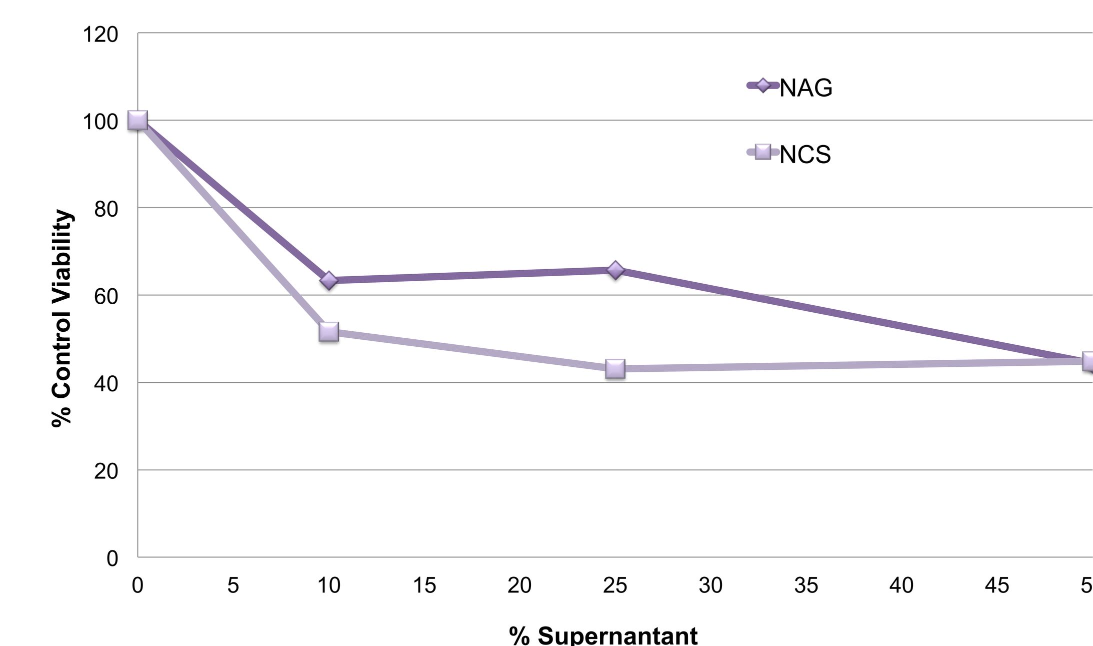

Fig 3. Supernatant from *P. uticensis* effects cell growth of *Candida albicans*. *C. albicans* yeast cells were inoculated at 10^6 cells/ml with increasing concentrations of *P. uticensis* supernatant. Cells were incubated at 37°C for 1 hr. After incubation, cells were stained with PrestoBlue Cell Viability reagent. Cells were centrifuged and resuspended in minimal media. A cell viability assay was performed using a fluorescent microplate reader. Each point represents the average percent of control viability. Cell viability decreases as supernatant concentration increases.

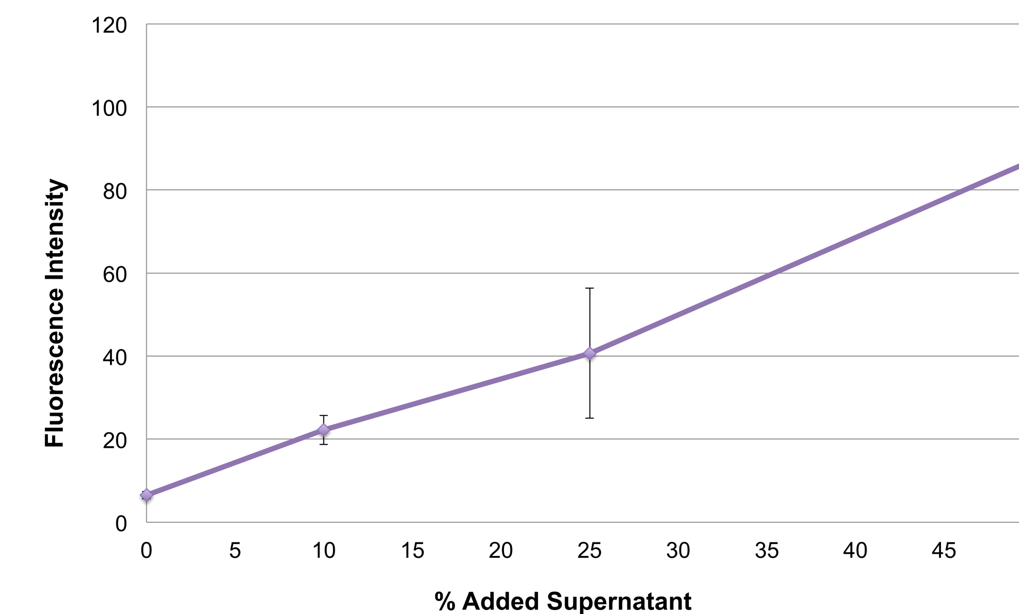

Fig 4. Supernatant from *P. uticensis* effects cytoplasmic Ca^{2+} levels. Yeast cells were inoculated at 10^6 cells/ml with increasing concentrations of *P. uticensis* supernatant. Cells were incubated at 37°C for 1 hr, then stained with chlorotetracycline (CTC) for 30 min. Cells were centrifuged and resuspended in minimal media. Cytoplasmic Ca^{2+} levels were determined using a fluorescent microplate reader. Each point represents the average fluorescence intensity . As concentration of supernatant increases, an increase in concentration of cytoplasmic calcium is shown suggesting that one of the antifungal secretions of *P. uticensis* is a calcium ionophore.

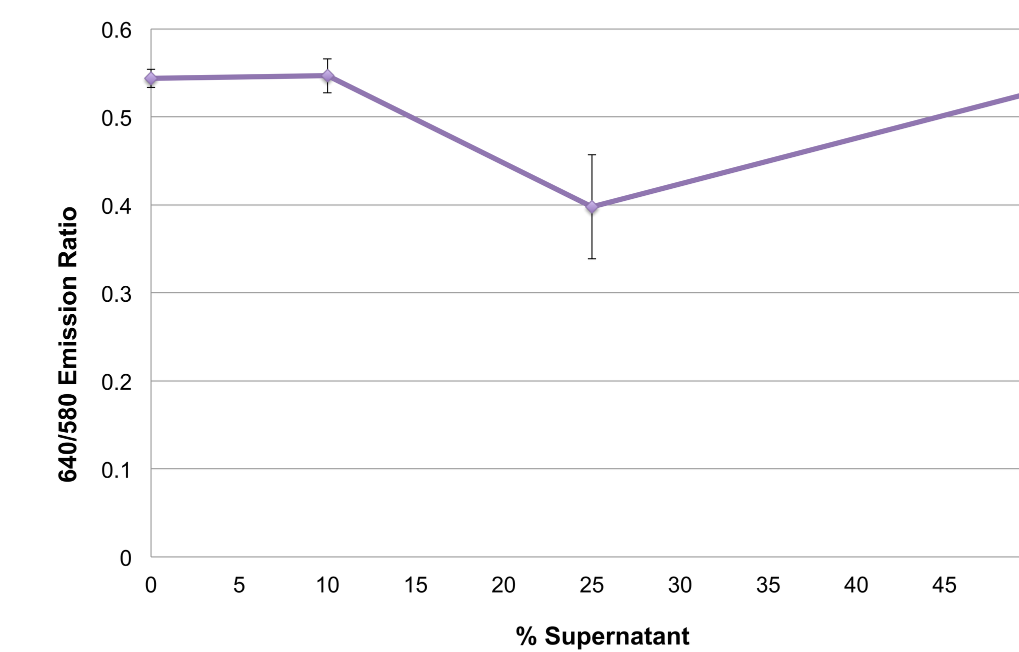

Fig. 5. Supernatant from *P. uticensis* effects cytoplasmic pH. *C. albicans* yeast cells were inoculated at 10^6 cells/ml with increasing concentrations of *P. uticensis* supernatant. Cells were incubated at 37°C for 1 hr. Cells were stained with C-SNARF and incubated for an additional 30 min. Cells were centrifuged and resuspended in minimal media. Cytoplasmic pH was computed using a fluorescent microplate reader. Each point represents the average emission ratio at 640 nm and 580 nm. Results are inconclusive.

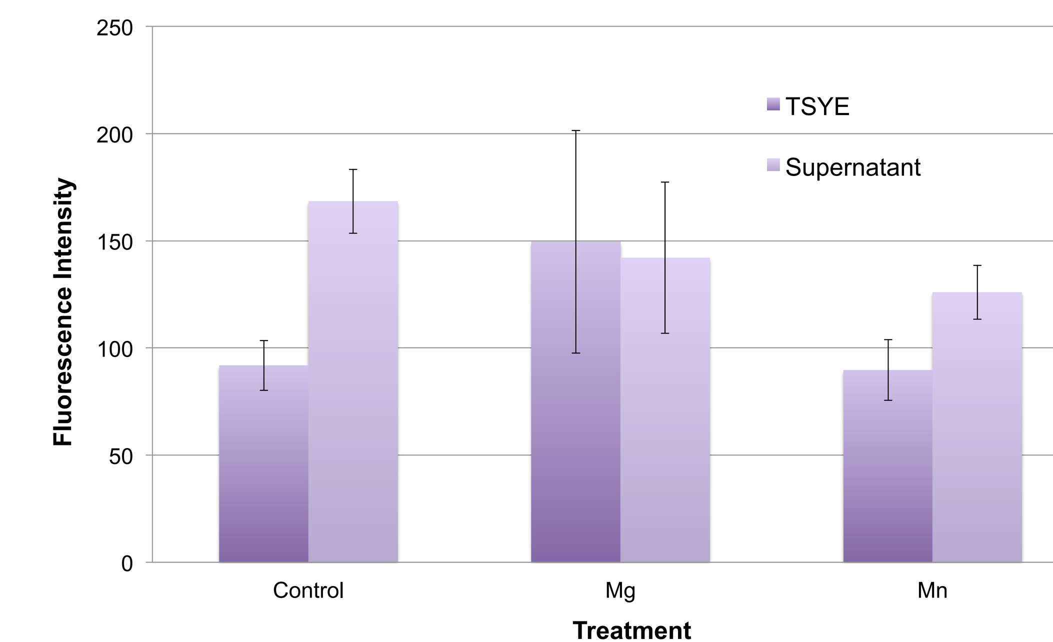

Fig. 6. Supernatant from *P. uticensis* effects calcium flux inhibition. : *C. albicans* yeast cells were inoculated at 10^6 cells/ml with increasing concentrations of *P. uticensis* supernatant. Two sets of tubes were prepared; one set containing the bacterial supernatant and the other a control set. $MgCl_2$ or $MnSO_4$ was added to tube sets and then cells were incubated for 1 hr at 37°C. Cells were stained with CTC and incubated for an additional 30 min. Cells were centrifuged and resuspended in minimal media. Fluorescence activity was measured using the fluorescence microplate reader. Each point represents the average fluorescence intensity. The graph demonstrates that the Mg^{2+} ion does not effect either the supernatant nor the control. The Mn2+ ion reduces fluorescence intensity in supernatant showing evidence of blockage.

CONCLUSION

Based on the results of these studies, we conclude that increasing concentrations of *P. uticensis* supernatant results in a reduction in cell hydrophobicity, cell growth, and cell viability. Cytoplasmic Ca^{2+} and calcium flux inhibition experiments suggest that a calcium ionophore is present in the antifungal compounds found in *P. uticensis*. This is supported by the increased cytoplasmic calcium levels due to Ca^{2+} influx observed with increasing *P. uticensis* supernatant concentrations, and inhibition of this influx due to competition with Mn^{2+} ions. However, we failed to see evidence of inhibition of the alkalinization of the cytoplasm reported to occur with the onset of hyphal formation.

LITERATURE CITED

Benzing, S.L. Cotrupe, C.C. and Aaronson, L.R. 2012. Light Effects on Pyomelanin Production in a Novel *Pseudomonas* Species. Abstracts of the 112th General Meeting of the American Society for Microbiology.

Berman, J., Gow, N.,Sudbery, P. 2004. The distinct morphogenic states of Candida albicans. TRENDS in Microbiology. 12: 317-324.

Gerlach, J.A., Bart, K.M., and Aaronson, L.R. 1996. Sphinganine alters growth, morphology and metabolite transport in *Candida albicans*. Abstr.of the 96th Gen. Mtg. of the Amer. Soc. Microbiol. pg 544.

Ha, K. C., and T. C. White. 1999. Effects of azole antifungal drugs on the transition from yeast cells to hyphae in susceptible and resistant isolates of the pathogenic yeast *Candida albicans. Antimicrob. Agents Chemother.* 43: 463-468.

Haoping, L. 2001. Transcriptional control of dimorphism in Candida albicans. Growth and Development. 4: 728-735.

Hube, B., Mayer, F.M., Wilson, D. 2013. Candida albicans pathogenicity mechanisms. Virulence. 4: 119-128.

Jacobsen, I.D., D. Wilson, B. Wachtler, S. Brunke, J. Naglik, and B. Hube. 2012. *Candida albicans* dimorphism as a therapeutic target. *Expert Rev. Anti Infect. Ther.* 10: 85-93.

Kracke, K.M., and Aaronson, L.R. 2011. Photoinduction of Pyomelanin Production in a Novel *Pseudomonas* Species. Abstracts of the 112th General Meeting of the American Society for Microbiology.

Larson, R.A., Wilson, M.M, .Guss, A.M, and Metcalf, W.W. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria Arch. Microbiol. 178 :193–201.

Seifert, S.L., Shikula, K., and Aaronson, L.R. 2013. Pyomelanin Production in a Novel *Pseudomonas* Species is Cell Density-Dependent. Abstracts of the 113th General Meeting of the American Society for Microbiology.

van Kessel, J.C., Scanlon, T.L., and Aaronson, L.R. 2003. Identification of the cutaneous antifungal microbial flora of the red-backed salamander, *Plethodon cinereus*. *Abstracts of the 103rd Gen. Mtg. Of the Am. Soc. for Microbiol.* pg. 435.

Vogel, H.J. 1956. A convenient growth medium for *Neurospora* (medium N). *Microbiol. Genet. Bull.* 13: 42-43.

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