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V. H. Nguyen, M. Alexeyev,
N. Kozyrovska, V. Kordyum, J. Elhai

ANABAENA THERMALIS: A NITROGEN-FIXING CYANOBACTERIUM ASSOCIATED WITH RICE

The nitrogen-fixing filamentous cyanobacterium A. thermalis SpA was isolated from the surface of wild rice growing in Viet Nam. Electron microscopy shows that the purified strain is able to penetrate into the root interior of surface sterilized rice plants. Attempts have been initiated to genetically manipulate A. thermalis SpA in order to understand whether nitrogen fixation and adherence are critical factors underlying the reported ability of the strain to enhance rice yields. As a first step, we have shown that A. thermalis SpA does not possess a DNA restriction-modification system that might limit the introduction of foreign DNA into the strain.

Cyanobacteria have traditionally been applied to rice paddies of southeast Asia. Inoculation of rice grain with nitrogen-fixing cyanobacteria has been shown in certain cases to increase the yield of rice in the absence of mineral fertilizers [1]. There are historical accounts describing the agricultural use of cyanobacteria in symbiotic association with the water-fern Azolla as early as 2000 years ago in China and 900 years ago in Viet Nam [2]. Despite the high productivity of rice fields on which Azolla is applied as a green manure, the practice is not widely employed, owing both to its high labor costs and to the sensitivity of the fern to environmental factors [3].

There have been numerous reports concerning the beneficial effect of inoculating rice paddies with free-living cyanobacteria, but many other trials were unable to demonstrate an effect [1]. The cyanobacterial property responsible for the beneficial effect, when it occurs, is not known, although it is often presumed to be the ability to fix nitrogen. Cyanobacteria indigenous to rice fields are recognized to be of importance in maintaining the fertility of the field [4]. In this regard, nitrogen fixation is of prime importance [5], but other factors could also be important, such as the production of phytohormones and vitamins [1, 6].

The success of inoculation may depend on several factors, and certainly one important factor is the ability of the cyanobacterium to survive in the natural environment. Adherent cyanobacteria may possess a special advantage towards survival in that cells attached to the plant may provide an inoculum to regenerate the population of epiphytic cells lost due to flooding [7, 8]. Likewise, the ability of certain epiphytic cyanobacteria to invade the interior of the plant [8] may provide the means to recolonize the surface of a plant and thus withstand predation. Epiphytic cyanobacteria are of particular interest also in that nitrogen fixed by them might be more readily taken up by plants than nitrogen fixed by free-living cyanobacteria.

Recent advances in molecular biology provide promising ways to improve and expand the useful properties of microorganisms for practical purposes and powerful tools with which to understand the basis of their beneficial interactions with plants. Specifically, the alteration of cyano-

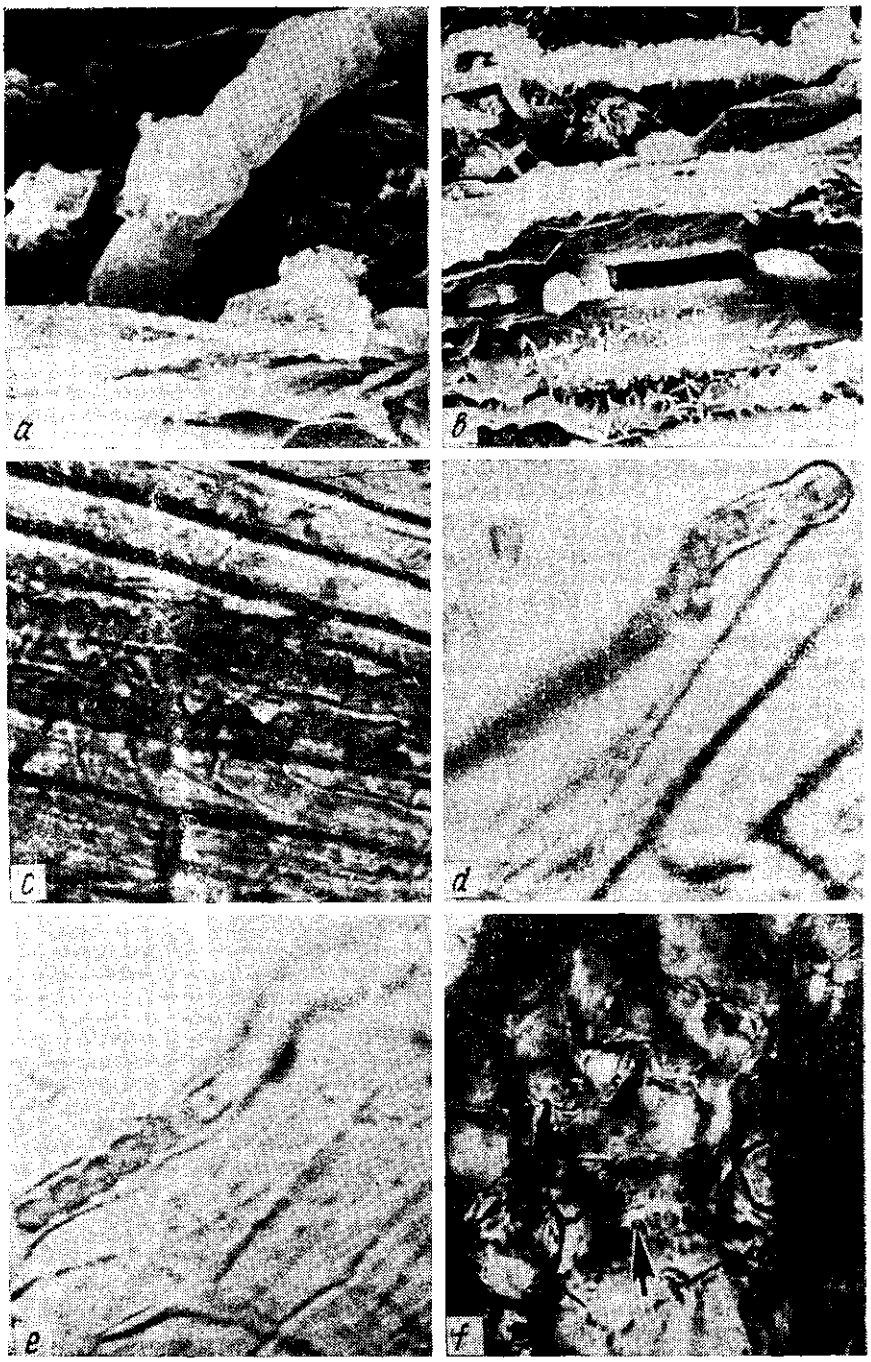

Fig. 1. Localization of cyanobacteria in the interior of rice plants: *a*, *b* — within stoma, *c* — within tha xylem of roots; *d*, *e* — within root hairs; *f* — within aerenchyma of roots. X 5400 (*a*); 4800 (*b*); 2400 (*c* — e); 800 (*f*)

bacteria in certain characteristics (e. g., nitrogen fixation and adhesiveness) will allow us to test whether those characteristics are critical in enhancing rice yields.

Eleven strains of nitrogen-fixing cyanobacteria were isolated from the stems of wild rice growing in nitrogen-limited marshes in Viet Nam. They were purified and scored on the basis of their ability to stick to rice stems in the laboratory. The most adherent strain, identified as *A. thermalis* (strain SpA), was chosen for further experiments.

A. thermalis SpA grows on agar media as spots consisting of filaments that are very gentle at first and later look like a crust. Often filaments form branched and anastomosed stacke. Trichomes are straight or slight curved and arranged close to one another. Young trichomes are a bit narrowed at the ends, and sometimes one end of a trichome is narrow and the opposite end has a heterocyst.

The association between *A. thermalis* SpA and rice was assessed in the laboratory by microscopic observation of rice plants inoculated with cyanobacterial cultures. Surface sterilized rice seeds (strains IR-64, IR-8, Taipei-2, 8423 from International Rice Research Institute, Los Banos, Philippines) were inoculated with a 20 day-old culture of *A. thermalis* SpA and other cyanobacterial isolates. Thirty days later, tissue from rice

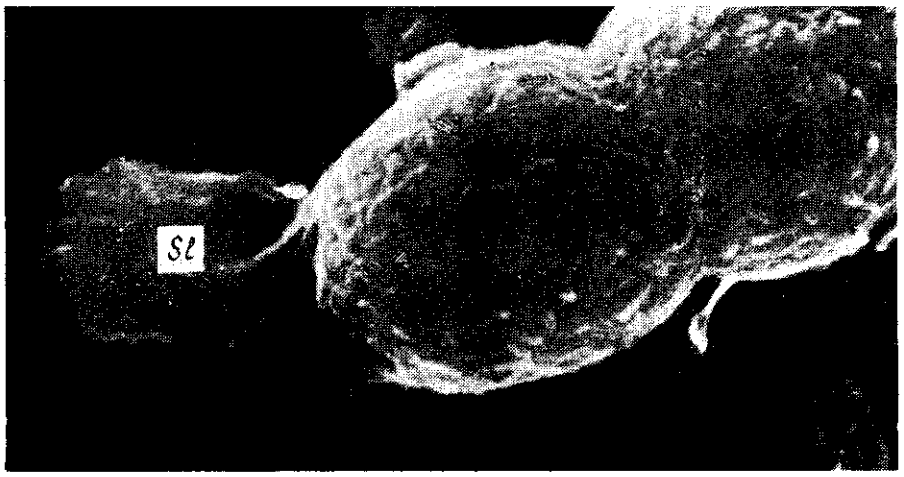

Fig. 2. Adesion of cyanobacterial trichome to the leaf surface; *Sl* — slime. ×13000

seedlings was examined. Trichomes of cyanobacteria were found within stoma (Fig. 1, *a*, *b*) and in root xylem (Fig. 1, *c*) of plants inoculated with *A. thermalis* SpA. Cyanobacteria were never observed within plants inoculated with the other ten cyanobacterial isolates.

Filaments observed within plant tissue did not differ appreciably from those of *A. thermalis* SpA grown in culture. Perhaps the most interesting observations were of cyanobacterial chains (3—14 cells) within root hairs (Fig. 1, *d*, *e*). These were rarely observed but the finding was confirmed in several independent cocultivation experiments, especially those using rice strains IR-64, IR-8, and Taipei-2. Cyanobacterial trichomes were also seen within the aerenchyma of roots (Fig. 1, *f*). Scanning micrographs show that *A. thermalis* SpA adheres well to the surface of leaves, stems, and roots (Fig. 2).

The abilities of *A. thermalis* SpA to adhere to the surface of the rice plant and to penetrate into root tissue suggest that the strain may be particularly capable of surviving competition and predation in foreign rice fields. In contrast, free-living cyanobacteria inoculated into foreign rice fields generally survive poorly [9, 10]. In order to identify *A. thermalis* SpA after inoculation and thereby monitor its survival in the field, we are attempting to tag the starin with a reporter gene absent from natural cyanobacterial strains. This strain will be used to assess whether inoculation is an effective means of altering the natural population of cyanobacteria associated with rice.

Inoculation of rice seeds with *A. thermalis* SpA has been shown to increase the yield of rice by 12 % [8]. Understanding what properties of the strain contributes to the increased yield may help us see how to increase yields further, either by modifying the cyanobacterium or altering agricultural practices. Mutants of *A. thermalis* SpA defective in dif-

ferent characteristics of potential benefit to the plant may lead to a theoretical foundation for the observed increase in rice yield by inoculation. Genetic manipulation of cyanobacteria requires the ability to transfer DNA into their cells. One obstacle to intergeneric DNA transfer into cyanobacteria is the occurence of sequence-specific restriction endonucleases [11, 12].

Our first step to approach the genetic manipulation of *A. thermalis* SpA was to test this cyanobacterium for the presence of restriction-modification systems. Exponentially growing cultures (10 ml) of *A. thermalis* SpA were sedimented and washed with buffer A (50 mM NaCl, 7 mM 2-mercaptoethanol, 20 mM tris-HCl, pH 7.5). The resulting pellet was resuspended in 500 μl of the same buffer and sonicated on ice with a disintegrator («MSE», UK) six times in 10 second bursts at maximum power. Cell debris was removed by centrifugation (10 000 *g*, 4 °C) and 3 μl of the supernatant were incubated with 1 μg of DNA isolated from phage lambda, in 20 μl of 50 mM tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, at 37 °C for 20 min. Restriction fragments were separated by electrophoresis in 0.8 % agarose. Strain *Anabaena sp.* PCC 7120, which carries isoschizomers of *R. AvaI*, *R. AvaII*, and *R. AvaIII*, was used as a positive control. Specific endonuclease activity was not observed in cell-free extracts of *A. thermalis* SpA, but extracts of *Anabaena sp.* PCC 7120 showed the expected activities (Fig. 3). The absence of the restriction-modification systems in *A. thermalis* SpA should facilitate genetic manipulation of the strain.

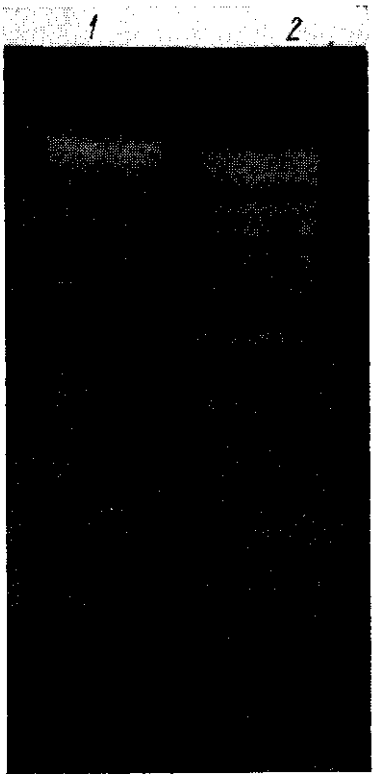

Fig. 3. Electrophoretic pattern of DNA from phage lambda, digested with crude lysates from (*1*) *A. thermalis* SpA or (2) *Anabaena sp.* PCC 7120

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М. Ф. Алексеев, Г. Л. Ковтунович, А. Н. Кравец, А. С. Солонин

ВЕКТОРЫ КЛОНИРОВАНИЯ ДЛЯ ESCHERICHIA COLI И ЭНДОРИЗОСФЕРНОГО АЗОТФИКСАТОРА KLEBSIELLA OXYTOCA VN13 НА ОСНОВЕ РЕПЛИКОНА ПРИРОДНОЙ HSD ПЛАЗМИДЫ pZE8

На основе репликона природной hsd плазмиды Citrobacter freundii сконструированы векторные плазмиды для K. oxytoca VN13 и E. coli. Векторы pKAS18 и pKAS19Б имеют селективный маркер устойчивости к канамицину и полилинкеры плазмид pUC18 и pUC19 соответственно. Плазмиды pMG1k и pMG21k предназначены для клонирования в уникальном PstI сайте гена рестриктазы EcoRV.

Введение. В связи с обострившейся экологической ситуацией все большую актуальность приобретают исследования в области генетики азотфиксирующих микроорганизмов. Понимание молекулярных механизмов азотфиксации и взаимодействия бактерий с растениями позволит решить проблему связанного азота путем создания экологически чистых бактериальных удобрений. Использование для этих целей генетически измененных свободноживущих и эндоризосферных азотфиксирующих микроорганизмов представляется нам весьма перспективным.

Ранее были выделены азотфиксирующие энтеробактерии *K. oxytoca* VN13, обладающие двумя уникальными свойствами:

1) будучи способными колонизировать сосудистые пучки растений, они занимают уникальную экологическую нишу, что делает их исключительно конкурентоспособными по отношению к почвенной микрофлоре [1];

2) эти бактерии выделяют естественный стимулятор роста растений — индолил-3-уксусную кислоту [2].

Полевые испытания показали, что сочетание этих двух свойств повышает урожай на 100 % при обработке семян перед посевом суспензией бактерий. Генетическое изменение полезных свойств *K. oxytoca* VN13 в сторону их усиления предполагает создание эффективной системы клонирования генов в этом организме, и, в первую очередь, конструирование высокостабильных векторов, обладающих удобными селективными маркерами, чему и посвящена данная работа.

Материалы и методы. В работе использованы: штаммы *E. coli* JM109 recA1, endA1, hsdR17, gyrA96, thi, supE44, relA1, λ-, Δ(lac-proA, B), F′, traD36, proA, B, lacIqZΔM15 [3]; *E. coli Z85*Δ(lac-proA, B),