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Nitrapyrin-ammonium combination induces rapid multiplication of mixed cultures of the stalked bacterium *Nevskia ramosa* famintzin and other heterotrophic bacteria

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Abstract. *Nevskia ramosa*, a bacterium that is very difficult to grow in artificial culture media in its recognizable and natural habit was induced to grow and multiply rapidly in mixed culture in an amended ASM medium. ASM liquid medium, an inorganic mineral medium, amended with 0.5 μg ml^{-1} of nitrapyrin and various levels of ammonium chloride (25–100 μM) produced very significant enrichment of *Nevskia ramosa* within 24 h, permitting formation of its characteristic bushy dichotomously branched, stalked colonies with the bacterial cells at the apices of branches. ASM medium amended with either nitrapyrin or ammonium singly failed to produce growth of *N. ramosa*. The nitrapyrin-ammonium combination in ASM medium also produced a significant increase in growth of other heterotrophic bacteria. Our results should be of value to bacteriologists interested in studying *N. ramosa*, a species which has received little attention thus far.

Key words: Stalked bacteria – *Nevskia* – Nitrapyrin – N–serve – Aquatic bacteria – Culture medium

Nitrapyrin or N-serve (2-chloro-6-(trichloromethyl) pyridine) is a specific inhibitor of the ammonium oxidation component of nitrification (Hughes and Welch 1970; Hendrickson and Keeney 1979; Zacherl and Amberger 1990). Nitrapyrin lyses nitrifying bacteria at concentrations above 1 mg l^{-1}, and at low concentrations (~0.5 mg l^{-1}) chelation of nitrification enzymes is suspected (Benmoussa et al. 1984). Nitrapyrin was found to inhibit mostly chemoautotrophic microorganisms (Priscu et al. 1990; Benmoussa et al. 1984), and appears to be most effective under specific environmental conditions (Hendrickson and Keeney 1979). We are not aware of any reports showing bacterial (heterotrophic or chemoautotrophic) growth enhancement by nitrapyrin whether singly or in combination with other chemicals.

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Our studies showed rapid multiplication of both gram positive and gram negative heterotrophic bacteria induced by nitrapyrin in conjunction with ammonium in ASM^- medium (ASM medium free of N-source) (Allen 1968). The most intriguing observation was the nitrapyrin-induced rapid growth of *Nevskia ramosa* (in its stalked form) a bacterium that is difficult to grow into stalked colonies under artificial growth conditions (Babenzien 1989; Heldal and Tumyr 1986; Hirsch 1981). ASM^- medium, with the appropriate level of nitrapyrin-ammonium combination, would therefore be a very useful culture medium for *N. ramosa* and other species of bacteria.

Materials and methods

Culture and experimental conditions

ASM medium (Allen 1968) without inorganic N (ASM^-) at pH 8 ± 0.1 was amended with 0, 25, 50 or 100 μM NH_4Cl. Three replicate conical flasks (125 ml) each containing 40 ml of the above 4 media were again amended with either 0 or 0.5 μg ml^{-1} nitrapyrin dissolved in 95% ethanol. Flasks containing no nitrapyrin were treated with the same volume (8 μl) of 95% ethanol as the nitrapyrin solution. The flasks were then inoculated with 0.5 ml bacterial suspension in ASM medium obtained by washing a surface bloom of the cyanobacterium *Anabaena flos-aquae* from Hebgen Lake, Montana. Before inoculation the bacterial suspension was passed through a membrane filter with 8 μm pore size to remove cyanobacterial filaments. The flasks were incubated at 25 ± 1 °C on a gyrotary shaker at 60 rpm.

ASM medium contains, per liter 1 ml each of 0.10 M $Na_2HPO_4 \cdot 7\,H_2O$, 0.10 M K_2HPO_4, 0.19 M $MgSO_4 \cdot 7\,H_2O$, 0.375 M $MgCl_2 \cdot 6\,H_2O$, 0.20 M $CaCl_2 \cdot 6\,H_2O$, 0.02 M $Na_2EDTA \cdot 2\,H_2O$ + 0.004 M $FeCl_3 \cdot 6\,H_2O$, and 1 ml of the trace element mixture containing 0.0524 M H_3BO_3, 0.0106 M $MnSO_4 \cdot 5\,H_2O$, 0.00104 M $ZnSO_4 \cdot 7\,H_2O$, 0.00045 M $Na_2MoO_4 \cdot 2\,H_2O$, 0.0002 M $CuSO_4 \cdot 5\,H_2O$, 0.0005 M $CoCl_2 \cdot 6\,H_2O$, 0.0020 M $AlCl_3 \cdot .6\,H_2O$, 0.000645 M $NiSO_4$, and 0.000542 M KI. Na_2HPO_4 and K_2HPO_4 mixed up in 500 ml double distilled water was pH adjusted to 8.15 after adding 25 mg tricine buffer. This mixture was autoclaved separately from the other 500 ml mixture containing all the other components, and combined at room temperature.

Bacterial enumeration

Enumeration of total and stalked bacteria (*N. ramosa*) was carried out at the onset of the experiment, on day 1, day 2 and day 3; 500 µl sample per flask was treated with 5 µl of 0.05% (w/v) aqueous acridine orange, and incubated at room temperature for 30 min before filtering at 12 cm Hg vacuum through 0.2 µm polycarbonate filters pre-stained with Irgalan black. The filters were air dried for about 10 min before placing on a microscope slide. A drop of immersion oil was added to the filter before adding a cover slip. A counting grid mounted on the eye piece of an epifluorescence microscope (Nikon Labophot, Tokyo, Japan, fitted with a Nikon DM 510 B-3A filter cube) was used to make 3 separate bacterial cell counts per sample (for both total and stalked bacteria ml^{-1}) according to the procedure of Hobbie et al. (1977). Means of 3 counts (converted to cell number ml^{-1}) per sample (3 replicates) were then analyzed using analysis of variance to determine statistical differences among treatments.

Examination of the stalked bacteria for identification

The stalked bacteria on a glass slide were treated with 10% (w/v) aqueous solution of potassium ferrocyanide, and potassium ferricyanide respectively and incubated for 10 min before microscopic examination. There were no shades of blue or bluish-green staining of the bacterial stalk indicating absence of detectable levels of ferrous or ferric iron deposits in the stalk. Gram staining was carried out separately on both stalked and other bacteria in the cultures at 24 h. Measurements of length and thickness of the individual bacterial cells at the tips of stalks, and the diameter of their colonies were made using a calibrated micrometer eye piece.

Results

Varios levels of ammonium without nitrapyrin, nitrapyrin in the absence of ammonium, and controls with neither nitrapyrin nor ammonium failed to show *N. ramosa* growth up to 3 days of incubation (several samples incubated for up to 5 days gave the same results). Different levels of ammonium combined with nitrapyrin (0.5 µl ml^{-1} concentration) produced very significant growth of *N. ramosa* within 24 h (Table 1) in enrichment cultures. Total bacterial growth, even though enhanced by ammonium alone in the ASM^- medium, and inhibited by nitrapyrin alone in the same medium (compared to control), was significantly higher in treatments containing both nitrapyrin and ammonium (Table 2). The mixed bacterial culture contained both gram positive and gram negative bacteria.

The habit of *N. ramosa* in our experiments (Fig. 1) had all the characteristics described and shown by plates in Bergey's Manual of Systematic Bacteriology (Babenzien 1989). The colony size was variable and the largest averaged about 60–70 µm diameter. Dimensions of individual cells at the tips of stalks varied to some extent but on average they were 2.1 µm long and 0.6 µm broad. They showed negative gram reaction. The cell dimensions we observed did not tally with the original descriptions of *N. ramosa* by Famintzin (1892) but were very close to the dimensions of *N. ramosa* cells described by Hirsch (1981). The bacterial stalks failed to show any visible

Table 1. Effect of various amendments to ASM^- medium on multiplication (cell number ml^{-1}) of *Nevskia ramosa* (Mean ±SE, $n = 3$)

Treatment[a]	Day 0	Day 1	Day 2	Day 3
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	$3.5 \times 10^4 \pm 5.7 \times 10^3$	$2.5 \times 10^5 \pm 3.8 \times 10^4$	$4.9 \times 10^5 \pm 8.5 \times 10^4$
7	0	$2.6 \times 10^5 \pm 3.0 \times 10^4$	$1.2 \times 10^6 \pm 3.3 \times 10^5$	$5.2 \times 10^6 \pm 1.4 \times 10^6$
8	0	$3.9 \times 10^5 \pm 4.3 \times 10^4$	$3.7 \times 10^6 \pm 1.8 \times 10^5$	$3.0 \times 10^7 \pm 1.2 \times 10^7$

[a] Treatment contents in addition to ASM^- were as follows: 1. no ammonium, no nitrapyrin, 2. 25 µM ammonium, no nitrapyrin, 3. 50 µM ammonium, no nitrapyrin, 4. 100 µM ammonium, no nitrapyrin, 5. no ammonium, 0.5 µg ml^{-1} nitrapyrin, 6. 25 µM ammonium, 0.5 µg ml^{-1} nitrapyrin, 7. 50 µM ammonium, 0.5 µg ml^{-1} nitrapyrin, 8. 100 µM ammonium, 0.5 µg ml^{-1} nitrapyrin

Table 2. Effect of various amendments to ASM^- medium on multiplication (cell number ml^{-1}) of heterotrophic bacteria including *N. ramosa* (Mean ±SE, $n = 3$)

Treatment[a]	Day 0	Day 1	Day 2	Day 3
1	$1.3 \times 10^2 \pm 9.8$	$5.8 \times 10^2 \pm 92.9$	$4.1 \times 10^3 \pm 6.1 \times 10^2$	$7.4 \times 10^3 \pm 4.6 \times 10^2$
2	$1.3 \times 10^2 \pm 9.8$	$4.0 \times 10^4 \pm 4.6 \times 10^3$	$7.8 \times 10^3 \pm 7.0 \times 10^2$	$6.4 \times 10^4 \pm 9.8 \times 10^3$
3	$1.3 \times 10^2 \pm 9.8$	$3.6 \times 10^5 \pm 3.5 \times 10^4$	$7.5 \times 10^5 \pm 9.7 \times 10^4$	$9.7 \times 10^5 \pm 1.7 \times 10^5$
4	$1.3 \times 10^2 \pm 9.8$	$6.4 \times 10^5 \pm 8.4 \times 10^4$	$6.0 \times 10^6 \pm 6.1 \times 10^5$	$1.2 \times 10^7 \pm 2.7 \times 10^6$
5	$1.3 \times 10^2 \pm 9.8$	$2.9 \times 10^2 \pm 40.4$	$3.6 \times 10^3 \pm 6.0 \times 10^2$	$6.7 \times 10^3 \pm 4.6 \times 10^2$
6	$1.3 \times 10^2 \pm 9.8$	$7.8 \times 10^6 \pm 7.1 \times 10^5$	$1.2 \times 10^7 \pm 1.9 \times 10^6$	$3.8 \times 10^8 \pm 1.5 \times 10^7$
7	$1.3 \times 10^2 \pm 9.8$	$4.2 \times 10^7 \pm 5.0 \times 10^6$	$2.9 \times 10^8 \pm 2.2 \times 10^7$	$9.0 \times 10^8 \pm 1.4 \times 10^8$
8	$1.3 \times 10^2 \pm 9.8$	$2.6 \times 10^8 \pm 2.3 \times 10^7$	$7.4 \times 10^8 \pm 1.1 \times 10^8$	$2.1 \times 10^9 \pm 5.9 \times 10^8$

[a] Treatments are the same as in Table 1

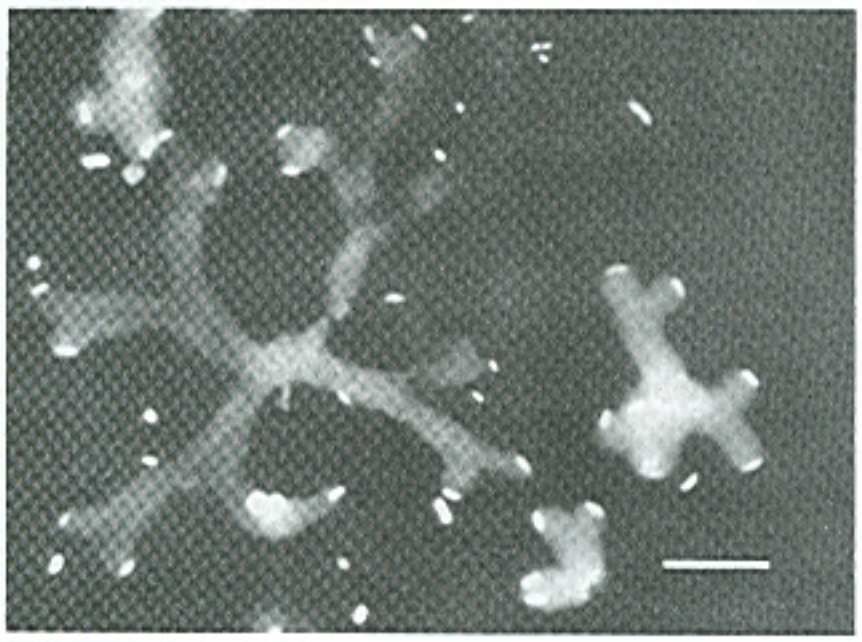

Fig. 1. Dichotomously branched stalked form of *Nevskia ramosa* induced to grow by ASM medium amended with nitrapyrin-ammonium combination. *Scale bar* = 10 μm

color change (under microscope) when treated with potassium ferrocyanide, or potassium ferricyanide.

Discussion

Our results suggest that neither nitrapyrin nor ammonium singly promote the growth or multiplication of *N. ramosa* in ASM medium. Combination of nitrapyrin and ammonium induces growth of this bacterium producing its characteristic stalked habit (Babenzien 1989; Heldel and Tumyr 1986; Henrici and Johnson 1935) in enrichment cultures. There was a maximum proportion of 2.1% *N. ramosa* on day 2 in the enrichment culture containing the lowest ammonium level with N-serve. This bacterium is known to be exceptionally difficult to grow in artificial media (Babenzien 1989; Heldal and Tumyr 1986; Hirsch 1981), therefore, even though we have not attempted to produce an axenic culture of this organism our culture medium should provide bacteriologists with a means of artificially growing *N. ramosa* within a short incubation period (1–3 days). Previously reported artificial methods to grow *N. ramosa* take about 10 days at 18–22 °C (Babenzien 1965) or 30–40 days at room temperature (Hirsch 1981). The bushy stalked habit of this organism observed in our studies (Fig. 1) is identical to the characteristics reported for *N. ramosa* (Babenzien 1989; Henrici and Johnson 1935; Hirsch 1981). We found this bacterium 1) to be gram-negative, 2) to lack ferrous or ferric iron deposits in the stalks thus eliminating the possibility of it being stalked iron bacteria (based on lack of color change with potassium ferro- and ferricyanide), 3) to have an average maximum colony dimension of 60–70 μm diameter, 4) to bear dichotomously branched acellular stalks and 5) to consist of individual cells which are elongated rods. All these characteristics conform to the previously described species *N. ramosa*. The cell dimensions reported previously vary considerably from report to report (Famintzin 1892; Babenzien 1965, 1967; Hirsch 1981); cell dimensions of our cultures were very similar to those reported by Hirsch (1981). Differences in cell dimensions may be due to either strain differences or cultivation conditions. On the basis of the unique morphological characteristics of *N. ramosa* (Babenzien 1989) there is very little doubt that the species we isolated was *N. ramosa*, the only recognized species in the genus.

Even though our results showed that ammonium alone in ASM$^-$ medium promotes, and nitrapyrin alone in the same medium inhibits multiplication of heterotrophic bacteria, the combination of nitrapyrin and ammonium in ASM$^-$ medium significantly promotes heterotrophic bacterial growth (Table 2). Because the combination of these two chemicals promotes the growth of both gram-positive and gram-negative heterotrophs, the combination may be useful to promote heterotrophic bacterial growth in general, particularly under conditions where complex components (e.g. proteins and carbohydrates) cannot be used.

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