Isolation of Nitrogen-Fixing Bacteria Containing Molybdenum-Independent Nitrogenases from Natural Environments

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Seven diazotrophs that grow well under Mo-deficient, N₂-fixing conditions were isolated from a variety of environments. These isolates fall in the γ subdivision of the class *Proteobacteria* and have genes that encode the Mo nitrogenase (nitrogenase 1) and the V nitrogenase (nitrogenase 2). Four of the isolates also harbor genes that encode the iron-only nitrogenase (nitrogenase 3).

Azotobacter vinelandii, an aerobic nitrogen-fixing soil bacterium, was the first diazotroph that was shown to have three distinct nitrogenases (4). One of these nitrogenases is the classical molybdenum (Mo)-containing nitrogenase (nitrogenase 1), while the other two are Mo-independent nitrogenases (nitrogenases 2 and 3). Nitrogenase 1 is expressed in N-free media containing Mo and has been extensively characterized (17, 22, 29). Nitrogenase 2 is a vanadium (V)-containing nitrogenase and is expressed in N-free, Mo-deficient medium containing V (9–12, 24). Nitrogenase 3 is an iron-only nitrogenase and is expressed in Mo- and V-deficient N-free medium (7).

The diazotrophs which have been shown to have Mo-independent nitrogenase systems include physiologically and phylogenetically diverse laboratory microorganisms, such as *Clostridium pasteurianum* (30), *Rhodobacter capsulatus* (25–27), *Anabaena variabilis* (16, 28), *Rhodospirillum rubrum* (8, 20), *Heliobacterium gestii* (18), and *Azospirillum brasilense* (6).

In this report we describe the isolation of nitrogen-fixing bacteria from environmental sources.

Strains DU1, TP1, WA1, and WC1 were isolated from mostly aquatic samples collected at sites located within a 25-mile radius of Raleigh, N.C. Strains SM1 and SM2 were isolated from a salt marsh located near Beaufort, N.C., and strain WB3 was isolated from a salt marsh located near Wrightsville Beach, N.C. Mo-deficient, N-free modified Burk medium (–Mo, –N BM) (2) containing 2% sucrose was used for growth and enrichment procedures. To isolate strains from aquatic sources, 8 ml of source water and 18 ml of deionized water were mixed with 0.75 ml of 40 $\times$ phosphate buffer (244 mM, pH 7.2) and 3 ml of 10 $\times$ Burk medium sucrose-salts (total volume, approximately 30 ml) in a 300-ml sidearm flask. To isolate an organism (strain WC1) from wood chip mulch, a spatulaful of wood chips was added to 30 ml of –Mo, –N BM. The initial enrichment cultures were incubated at 30°C with vigorous shaking for approximately 48 h or until a significant increase in turbidity was observed. One to three milliliters of each enrichment culture was transferred into 30 ml of fresh –N, –Mo BM and incubated as described above. For the second and third transfers in liquid medium, the procedure described above was repeated, except that the inoculum was diluted 100-fold (300 μ l of culture was added to 30 ml of fresh medium). One

hundred microliters of the third transfer culture was streaked onto –Mo, –N BM agar (containing 1.5% purified agar [BBL, Becton Dickinson]). After 2 to 4 days of incubation at 30°C, single colonies were streaked onto fresh agar medium for isolation. This procedure was repeated (usually four to six times) until each isolate appeared to be a pure culture, as judged by colony morphology and microscopic examination results.

The strains isolated from salt marshes (SM1, SM2, and WB3) grew as clumps in liquid media; therefore, all subsequent studies with these strains were performed on solid agar media. All of the strains were gram negative, catalase positive, and motile. None of the strains grew at 4°C, but they all grew at 13°C and the best growth occurred at temperatures near 30°C. Strains SM1 and SM2 grew on agar medium in the presence of 7% NaCl, whereas WB3, the only other salt marsh isolate, was able to grow in the presence of only 1% NaCl. Strains DU1, TP1, and WA1 grew in liquid Burk medium containing N at pHs ranging from 5.5 to 8.5, and strain WC1 grew at pHs ranging from 4.5 to 8.5. All of the growth parameters described above were determined under non-nitrogen-fixing conditions.

The approximate cell sizes, as determined by scanning electron microscopy, were as follows: DU1, 3.5 by 1.5 μ m; TP1, 2.2 by 1.6 μ m; WA1, 2.2 by 1.9 μ m; WB3, 2.2 by 1.7 μ m; WC1, 2.2 by 1.6 μ m; SM1, 1.2 by 0.6 μ m; and SM2, 1.2 by 0.6 μ m. It was interesting that DU1 appeared to divide by budding, as well as by binary fission.

16S rRNA genes were amplified and isolated as described by Barns et al. (1) by using universal primers 515F and 1492R (19). A phylogenetic analysis of these genes indicated that all of the isolates are members of the γ subdivision of the class *Proteobacteria* (Fig. 1) and that they appear to be specifically related to the fluorescent pseudomonads.

All of the strains grew on N-free agar medium in the presence of Mo or V and in the absence of Mo and V. Table 1 summarizes the results obtained for the four strains that grew without visible clumping in liquid media when growth was monitored with a Klett-Summerson colorimeter equipped with a no. 66 filter (red). Strains TP1 and WA1 grew diazotrophically in the presence of Mo or V or in the absence of these metals. These results suggest that isolates TP1 and WA1 utilize nitrogenase 1, 2, or 3. On the other hand, strains DU1 and WC1 grew in the presence of Mo or V but not in the absence of these metals. These results suggest that strains DU1 and WC1 have nitrogenases 1 and 2 but not nitrogenase 3. The finding that strains grew on N-free agar medium in the absence of Mo and V but not in liquid medium might be attributable to

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FIG. 1. Phylogenetic relationships between the environmental isolates and selected members of the *Proteobacteria*, as shown by an unrooted tree. Ten *Pseudomonas* strains used in the phylogenetic analysis are represented on the tree by *Pseudomonas stutzeri*. Bootstrap values (100 replicates) are shown at the branches for the environmental isolates; the values above the lines are DNA parsimony values, and the values below the lines are DNA maximum-likelihood values. Values less than 50% are not shown.

the presence of contaminating Mo or V in the purified agar. This possibility is consistent with the finding that strain CA70 (14), a mutant of *A. vinelandii* which lacks nitrogenase 3, grows on N-free agar medium in the absence of Mo and V but not in liquid medium having the same composition. Nitrogenase activity, as measured by acetylene reduction (3), was detected under all of the conditions which permitted diazotrophic growth and followed a pattern previously observed for *A. vinelandii* (4); i.e., cells grown in the presence of Mo gave significantly higher values than cells grown in the absence of Mo. The strains isolated from salt marshes (SM1, SM2, and WB3) exhibited nitrogenase activity when they were cultured on agar media in slants in the presence and absence of Mo or V, and as previously observed, the acetylene reduction values in the presence of Mo were considerably higher than the acetylene reduction values in the absence of Mo (results not shown). Nitrogenase activity was repressed by 10 mM NH_4^+ to different degrees for each of the three nitrogenase systems in the isolates (data not shown). An interesting difference between isolate TP1 and *A. vinelandii* is that TP1 did not grow in the presence of ammonium acetate at a concentration of 28 mM, the concentration ordinarily used for this nitrogen source in modified Burk medium. Preliminary results indicated that strain TP1 grows poorly or not at all in the presence of con-

TABLE 1. Diazotrophic growth and whole-cell nitrogenase activity in liquid media

Isolate	Addition to -Mo, -N BM	Generation time (h)	Nitrogenase activity (pmol of C_2H_4 /Klett unit $\cdot$ $\text{ml}^{-1} \cdot 30 \text{ min}^{-1}$)
DU1	NH_4^+ (10 mM)	2.5	— ^a
	Na_2MoO_4 (1 μM)	2.8	354
	V_2O_5 (1 μM)	3.6	34
	None	∞	—
TP1	NH_4^+ (10 mM)	2.2	—
	Na_2MoO_4 (1 μM)	2.1	844
	V_2O_5 (1 μM)	2.8	116
	None	3.2	66
WA1	NH_4^+ (10 mM)	2.2	—
	Na_2MoO_4 (1 μM)	2.6	524
	V_2O_5 (1 μM)	2.9	128
	None	3.2	54
WC1	NH_4^+ (10 mM)	2.0	—
	Na_2MoO_4 (1 μM)	2.4	287
	V_2O_5 (1 μM)	2.9	148
	None	∞	—

^a —, the measured activity was no greater than the activity of the blank (trichloroacetic acid-treated cells).

centrations of NH_4^+ greater than 14 mM regardless of the accompanying anion.

DNA fragments containing *A. vinelandii* *nifD* (0.8-kb *nifD* insert of pTMR18 [5]), *vnfD* (1.4-kb *vnfD* insert of pVDSJ1 [13]), and *anfD* (1.08-kb *anfD* insert of pPJD3A2 [23]) genes coding for the α subunits of nitrogenases 1, 2, and 3, respectively, were used as hybridization probes in Southern blot analyses of *EcoRI*-digested genomic DNAs of the isolates. Table 2 shows the sizes of the fragments which hybridized with the nitrogenase gene probes. *A. vinelandii* CA and *Pseudomonas fluorescens* DNAs served as positive and negative controls, respectively. The DNAs of isolates SM1, SM2, TP1, and WA1 yielded hybridizing fragments for each of the nitrogenase gene probes, suggesting that these isolates have representative genes for the three known nitrogenase systems in *A. vinelandii*. The presence of fragments in the strain DU1, WB3, and WC1 DNAs which hybridized to the *nifD* and *vnfD* probes suggests that these strains have genes that encode nitrogenases 1 and 2. The DNAs of these three strains, however, lacked fragments

TABLE 2. DNA fragments in *EcoRI* digests of genomic DNAs hybridizing to nitrogenase gene probes on Southern blots

Organism	Fragment size(s) (kb) with the following <i>A. vinelandii</i> nitrogenase gene probes:		
	<i>nifD</i>	<i>vnfD</i>	<i>anfD</i>
<i>A. vinelandii</i> CA	1.4, 10.0, 14.0	1.0, 2.6, 7.0	3.5, 4.0
<i>P. fluorescens</i>	ND ^a	ND	ND
DU1	1.4	1.8	ND
SM1	1.5	5.3	2.4
SM2	1.5	5.3	2.4
TP1	1.4	2.1, 4.7	22.6
WA1	1.4, 1.3	2.0, 4.7	2.7, 20.0
WB3	8.0, 9.0, 23.0	1.7, 4.5	ND
WC1	29.9	1.7, 4.6	ND

^a ND, DNA fragments hybridizing to nitrogenase gene probes were not detected.

FIG. 2. Comparisons of the amino acid sequences of VnfG (A) and AnfG (B). The amino acid sequences were deduced from the DNA sequences of PCR products and were aligned by using the Clustal-W algorithm. The sequences of *A. vinelandii* VnfG and AnfG were obtained from references 15 and 14, respectively.

that hybridized with the *anfD* gene probe. This observation is consistent with the inability of strains DU1 and WC1 to grow diazotrophically in liquid Mo-deficient medium lacking V, conditions which require nitrogenase 3 for diazotrophic growth (Table 1). The situation with strain WB3 is less clear since this strain does not grow well in liquid media, yet it grows and reduces acetylene on Mo-deficient agar medium in the absence of V. As mentioned above, it is possible that the purified agar contained trace amounts of Mo or V which could satisfy the requirements for nitrogenase 1 or 2.

For PCR amplification of *vnfG* and *anfG* (genes which encode the δ subunits of nitrogenases 2 and 3, respectively), conserved sites in *vnfD* and *anfD* were used as the priming sites for the forward PCR primers, and conserved sites in *vnfK* and *anfK* were used as the priming sites for the reverse primers. For amplification of *nifD*, conserved sites in *nifD* were used for

both the forward and reverse primers. Details concerning these primers and the PCR conditions used have been described previously (21). PCR products containing *vnfG* were obtained with genomic DNAs from all of the isolates, and the sizes were approximately 1.76 kb. Products (length, 0.49 kb) containing *nifD* were obtained from all of the isolates except strains WB3 and WC1. The only DNAs which yielded PCR products containing *anfG* were the DNAs of strains SM1, SM2, and TP1, and the product sizes were approximately 0.76 kb. The PCR results generally agree with the results obtained with Southern blots except that we were not able to detect *nifD* in strains WB3 and WC1 or *anfG* in WA1 when we used the PCR. The amino acid sequence data for the *vnfG* and *anfG* gene products shown in Fig. 2 indicate that there is a rather high degree of identity among these gene products in the isolates examined.

In this study we found that N_2 -fixing bacteria having the

ability to express Mo-independent nitrogenases can be readily isolated from environmental sources by using N-free enrichment media devoid of Mo. The ability to isolate these types of diazotrophs directly from environmental sources should expand our knowledge of diazotrophs that are able to express Mo-independent nitrogenases and should also provide information concerning the kinds of habitats in which these diazotrophs are found. The strains described in this report were isolated under highly aerobic conditions by using a medium containing sucrose as the carbon source. The use of microaerophilic and anaerobic enrichment conditions, as well as aerobic conditions, with a variety of carbon sources and medium formulations should increase our knowledge of the diverse diazotrophs that are known to be capable of using Mo-independent nitrogenases.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences of the nitrogen fixation genes are as follows: strain TP1 *nifD*, AF142477; strain TP1 *vnfDGK*, AF152908; strain TP1 *anfDGK*, AF152915; strain DU1 *nifD*, AF142478; strain DU1 *vnfDGK*, AF152909; strain WA1 *nifD*, AF152918; strain WA1 *vnfDGK*, AF152910; strain SM1 *nifD*, AF152920; strain SM1 *vnfDGK*, AF152911; strain SM1 *anfDGK*, AF152917; strain SM1 *nifD*, AF152919; strain SM2 *vnfDGK*, AF152912; strain SM1 *anfDGK*, AF152916; strain WB3 *vnfDGK*, AF152913; and strain WC1 *vnfDGK*, AF152914. The GenBank accession numbers for the 16S rRNA gene sequences are as follows: DU1, AF094765; WC1, AF094766; WB3, AF094767; WA1, AF094768; TP1, AF094769; SM1, AF094770; and SM2, AF094771.

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