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Metabolism of the Aliphatic Nitramine 4-Nitro-2,4-Diazabutanal by *Methylobacterium* sp. Strain JS178

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The aliphatic nitramine 4-nitro-2,4-diazabutanal (NDAB; $C_2H_5N_3O_3$) is a ring cleavage metabolite that accumulates during the aerobic degradation of the energetic compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by various *Rhodococcus* spp. NDAB is also produced during the alkaline hydrolysis of either RDX or octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and during the photolysis of RDX. Traces of NDAB were observed in a soil sampled from an ammunition-manufacturing facility contaminated with both HMX and RDX, suggesting natural attenuation. In this study, we report the isolation of a soil bacterium that is able to degrade NDAB under aerobic conditions. The isolate is a pink-pigmented facultative methylotroph affiliated with the genus *Methylobacterium*. The strain, named *Methylobacterium* sp. strain JS178, degrades NDAB as a sole nitrogen source, with concomitant growth and formation of 1 molar equivalent of nitrous oxide (N_2O). Comparison of the growth yield of strain JS178 grown on NDAB, nitrite (NO_2^-), or ammonium (NH_4^+) as a nitrogen source revealed that 1 N equivalent is assimilated from each mole of NDAB, which completes the nitrogen mass balance. In radiotracer experiments, strain JS178 mineralized 1 C of the [^{14}C]NDAB produced in situ from [^{14}C]RDX by *Rhodococcus* sp. strain DN22. Studies on the regulation of NDAB degradation indicated that allantoin, an intermediate in the purine catabolic pathway and a central molecule in the storage and transport of nitrogen in plants, up-regulated the enzyme(s) involved in the degradation of the nitramine. The results reveal the potential for the sequential participation of rhodococci and methylobacteria to effect the complete degradation of RDX.

The cyclic nitramines hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Fig. 1) are toxic energetic compounds that have caused severe soil and groundwater contamination (4). A good understanding of the microbial processes at contaminated sites is important in order to evaluate the fate of the compounds during natural attenuation. The mechanisms of transformation of RDX and HMX under anaerobic conditions have been well established (12). Although no aerobic bacteria are reported to degrade HMX, several aerobic bacteria have been isolated based on the ability to use RDX as a sole nitrogen source. Among these, three rhodococci have been reported: *Rhodococcus* sp. strain A (14), *Rhodococcus* sp. strain DN22 (5), and *Rhodococcus rhodochrous* strain 11Y (18). The initial denitration of the RDX molecule by rhodococci requires the involvement of a cytochrome P-450 (3, 6, 18). Denitration is followed by ring cleavage and the production of nitrous oxide (N_2O), ammonia (NH_3), formaldehyde (HCHO), and a dead-end product identified as 4-nitro-2,4-diazabutanal (NDAB) (Fig. 1) (8). The carbon stoichiometry clearly indicates that rhodococci mineralize 30 to 33% of the RDX (estimated as liberated CO_2) and that 62 to 64% of the remaining carbon accumulates as NDAB (8). NDAB is a transient intermediate during the degradation of HMX by the white-rot fungus *Phanerochaete chrysosporium* and is also produced during alkaline hydrolysis or photolysis of cyclic nitramines (10, 13). The widespread production of NDAB, via a variety of biotic and abiotic processes, underlines the environmental importance of this compound. Moreover, NDAB was present in soil contaminated with both HMX and RDX, signifying that its detection at contaminated sites might provide an effective method of monitoring natural attenuation (9). Because the aliphatic nitramine is highly soluble in water and does not sorb to soil particles, NDAB has a considerable potential to migrate offsite in surface waters or groundwater (9). This explains why NDAB was not reported to accumulate in soil. The results to date indicate that NDAB is degraded poorly, if at all, by soil microbes.

The genus *Methylobacterium*, belonging to the *Alphaproteobacteria*, is composed of a variety of pink-pigmented, facultatively methylotrophic bacteria that are capable of growth on one-carbon compounds (11). Here we report the isolation from soil of a *Methylobacterium* strain that is able to use NDAB as a sole N source and to mineralize the nitramine under aerobic conditions. Physiological and biochemical characteristics of the strain, as well as the metabolites produced from NDAB, have been determined. Allantoin up-regulated the ability of strain JS178 to degrade NDAB, suggesting that one or several amidohydrolases involved in the purine catabolism pathway could play a role in the degradation of the xenobiotic compound NDAB.

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MATERIALS AND METHODS

Organisms and growth conditions. The aerobic RDX degrader *Rhodococcus* sp. strain DN22 and *Methylobacterium* strain JS178 were cultivated in M-succi-

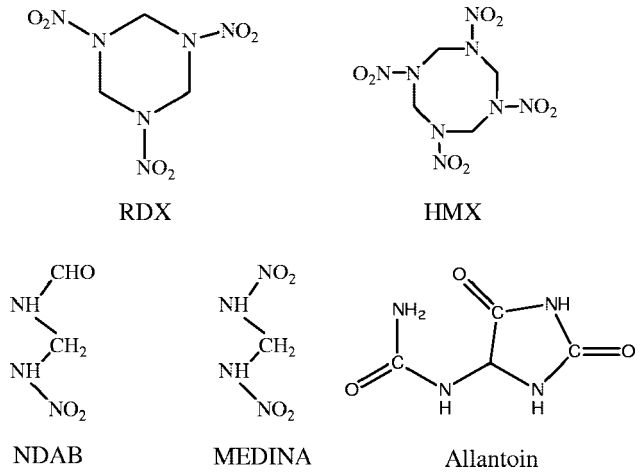

FIG. 1. Structures of RDX, HMX, NDAB, MEDINA, and allantoin.

nate (with 20 mM of succinate) as previously described (9). Bacteria were incubated at 30°C in a rotary shaker with agitation at 175 rpm.

Chemicals. RDX (>99% purity), [U-^{14}C]RDX (98% purity), HMX (99% purity), NDAB, and methylenedinitramine (MEDINA) were obtained as described previously (9).

Enrichment and isolation of aerobic bacteria able to degrade NDAB. A sample of sludge taken from an aerobic treatment plant at Tyndall Air Force Base (AFB), Fla., a garden soil from Washington, D.C. (pH 6.9), a garden soil from Lynn Haven, Fla. (pH 6.8), a forest soil from Tyndall AFB (pH 4.9), and an agricultural soil from a farm near Memphis, Tenn. (pH 7.8), were used as inocula (20% [wt/vol] soil slurries in water) for shake flask suspensions tested for the ability to degrade NDAB (100 μM). Slurries were incubated at room temperature under aerobic conditions, and NDAB concentrations were measured at intervals by high-performance liquid chromatography, as described previously (9). Suspensions in which NDAB disappeared were subjected to selective enrichment consisting of sequential transfer to M medium amended with 20 mM pyruvate and increasing concentrations of NDAB (100 to 400 μM) as the nitrogen source. Dilutions of the final suspension were plated on the above medium solidified with agarose (1.5%, wt/vol). Plates were incubated at 30°C, and a representative of the predominant small pink colonies was purified and designated strain JS178.

Mineralization assay. [^{14}C]NDAB was prepared from [^{14}C]RDX in M-succinate medium inoculated with RDX-induced strain DN22 (9). When the mineralization reached a plateau (30% of the radiolabel released as $^{14}CO_2$ and 64% remaining as [^{14}C]NDAB), DN22 cells were removed by filtration through a 0.22-μm-pore-size membrane (Millex-GP; Millipore, Bedford, Mass.) and the culture supernatant was inoculated with NDAB-grown *Methylobacterium* sp. strain JS178 to an initial A_{600} of 0.05. Parallel sets of microcosms were prepared with [^{14}C]RDX in M-succinate medium inoculated either with NDAB-induced strain JS178 alone or with both NDAB-induced strain JS178 and RDX-induced strain DN22. At each sampling, microcosms were aerated with 60 ml of 0.20-μm-filtered air (polytetrafluoroethylene membrane; Cole-Parmer, Vernon Hills, IL).

Growth of strain JS178 with NDAB as the sole N source. Cultures were performed in 10 ml of M-succinate medium with NDAB as the sole nitrogen source (150 μM or as indicated) in 125-ml serum bottles. The inoculum consisted of the addition of a washed stationary-phase JS178 culture grown in nutrient broth to an initial A_{600} of 0.04. The bottles were sealed with Teflon-coated septa and crimped with aluminum caps to allow the sampling of the headspace for N_2O analyses. The cultures were incubated as described above and were monitored for growth (A_{600}), residual NDAB, and possible metabolites such as nitrite (NO_2^-), nitrate (NO_3^-), formaldehyde (HCHO), methanol (CH_3OH), formate ($HCOO^-$), ammonium (NH_4^+), formamide (NH_2CHO), and nitramide (NH_2NO_2). Analyses were performed as described previously (9).

Resting-cell assays. Resting-cell assays were performed in sealed 6-ml vials containing M medium and NDAB (150 μM), without the addition of a carbon source. The biotransformation reaction (1 ml) was started with the addition (to a final A_{600} of 1.0) of strain JS718 (pregrown in M-succinate and various N sources, harvested at early to mid-log phase, and washed twice in M medium).

Molecular biology techniques. DNA was extracted from a JS178 colony grown on an NB plate, and the 16S rRNA gene was amplified and sequenced (2). Sequence comparisons were done using BLASTN (1).

Nucleotide sequence accession number. The 16S rRNA gene sequence of isolate JS178 was deposited in GenBank under accession number AY795971.

RESULTS AND DISCUSSION

Isolation of strain JS178. Selective enrichment was performed to isolate an aerobic bacterium that could use the RDX ring cleavage product NDAB as a nitrogen source. Among the several soil and wastewater sludge samples tested, the rate of NDAB disappearance in Tyndall AFB forest soil was twice that in the other soils (approximately 100 μM within 10 days). The Tyndall soil was chosen for the enrichment of an aerobic bacterium (strain JS178) that is able to grow using NDAB as the sole source of nitrogen. Strain JS178 is not the only microorganism that is able to transform NDAB (9), but it is the first that is able to grow at the expense of the nitramine.

Characterization of strain JS178. Strain JS178 grew on solidified M medium with succinate and NDAB, as pink-pigmented (nondiffusible) small colonies (diameter, 1 to 3 mm). The strain is gram negative, nonsporulating, rod-shaped, aerobic, and catalase and oxidase positive. In liquid culture, a ring of biomass formed around the wall of the incubation flask. Strain JS178 was able to grow on methanol as the sole C source. JS178 satisfied the morphological and biochemical criteria consistent with the genus *Methylobacterium*.

The 16S rRNA sequence comparisons revealed a close affiliation (99% similarity) of strain JS178 with *Methylobacterium populi* (19). A number of compounds, including substrates commonly used in the differentiation of *Methylobacterium* species (11), were tested as potential carbon sources. The results with strain JS178 were identical to those with *M. populi*. Both strains are able to use methanol, formate, acetate, betaine, ethanol, fructose, succinate, pyruvate, and methylamine (19). On the other hand, JS178 and *M. populi* did not grow on D-glucose, D-fucose, D-xylose, L-arabinose, or citrate.

Growth of strain JS178 on NDAB. In M-succinate medium with NDAB as the sole N source, the growth of JS178 was concomitant with the degradation of the nitramine and the production of N_2O (Fig. 2). No nitrite, nitrate, ammonium, nitramide, formaldehyde, formate, or formamide was detected during the degradation of NDAB by JS178. No methanol was detected, but since the initial concentration of NDAB was 150 μM, the maximum theoretical yield of methanol is 300 μM, which corresponds to the detection limit of the method used. The doubling time was approximately 1.8 days, and after 4 days of incubation, it significantly increased, probably due to oxygen limitation in the serum bottles. The degradation of NDAB and the production of N_2O were reflected in the growth kinetics, and the ratio of N_2O produced to NDAB consumed was 0.9 at both 4 and 7 days of incubation, clearly showing that approximately 1 mol of N_2O, or 2 N equivalents, was released per mol of NDAB degraded.

Number of nitrogen atoms assimilated from NDAB. The specific growth yields obtained with NDAB, $NaNO_2$, and $(NH_4)_2SO_4$ (Fig. 3) were 0.00173 (R = 0.999), 0.00165 (R = 0.995), and 0.00158 (R = 0.999) A_{600} unit/μmol of nitrogen substrate, respectively. The results clearly indicate that 1 N

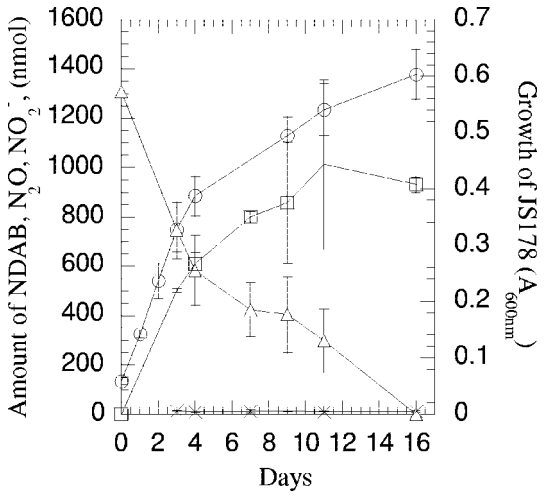

FIG. 2. Growth of *Methylobacterium* sp. strain JS178 in M-succinate and NDAB (1,300 nmol) as a sole N source. Shown are amounts of NDAB (△), N_2O (□), and NO_2^- (×). Growth is expressed as A_{600} (○). Error bars, standard deviations from duplicate experiments.

atom of a total of 3 in NDAB (Fig. 1) was assimilated during the growth of JS178.

Number of carbon atoms released from NDAB. The number of carbon atoms released during the degradation of NDAB was investigated as described previously (9). Briefly, [^{14}C]RDX was incubated in M-succinate with *Rhodococcus* sp. strain DN22 until approximately 30% of the radiolabel was liberated as CO_2. At this stage, RDX was totally depleted from the culture medium, and 92 to 96% of the [^{14}C]RDX added initially was transformed into [^{14}C]NDAB (9). A longer incubation period with strain DN22 did not result in more CO_2 production, even when more succinate (10 mM) was added to the medium (Fig. 4). The results confirm the inability of strain DN22 to degrade

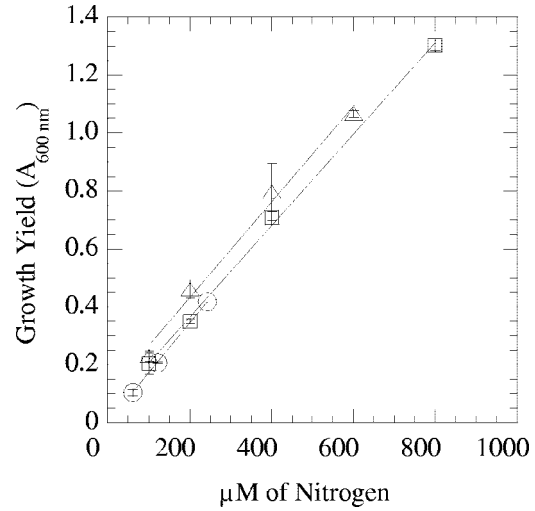

FIG. 3. Growth yield of *Methylobacterium* sp. strain JS178 in M-succinate, with nitrite (△), ammonium (□), and NDAB (○) as N sources. The linear regressions have the following gradients: 0.00165 (R = 0.995) for nitrite, 0.00158 (R = 0.999) for ammonium, and 0.00173 (R = 0.999) for NDAB. Error bars, standard deviations from triplicate experiments.

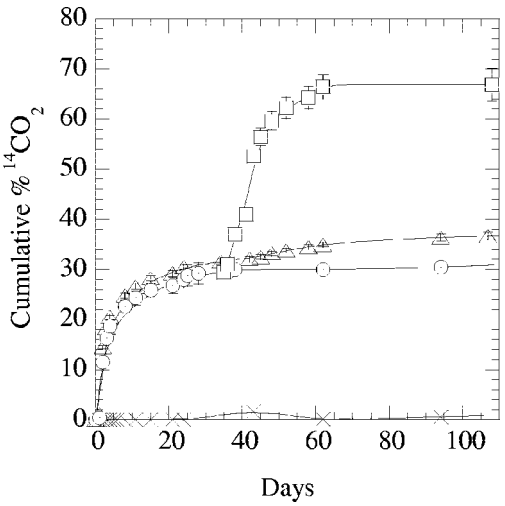

FIG. 4. Incubation of [^{14}C]RDX with *Rhodococcus* sp. strain DN22 (medium was supplemented with 10 mM succinate at day 25) (○), with *Methylobacterium* sp. strain JS178 (×), with both strains DN22 and JS178 (JS178 was reinoculated to the microcosms at day 42) (△), and with the sequential addition of strain DN22 (used to transform [^{14}C]RDX to [^{14}C]NDAB) and NDAB-grown JS178 (added when 30% of [^{14}C]CO_2 was liberated from [^{14}C]RDX, after the removal of DN22 cells by filtration) (□). Values are averages and standard deviations of duplicate experiments.

the NDAB produced. The incubation of [^{14}C]RDX with *Methylobacterium* sp. strain JS178 did not lead to production of CO_2, which indicated that it is unable to degrade RDX (Fig. 4). Compared to the incubation of [^{14}C]RDX with DN22, the simultaneous inoculation of M-succinate–[^{14}C]RDX medium with both strains DN22 and JS178 resulted in only a slight increase in CO_2 production. After the reinoculation of the microcosms with JS178 (performed at day 42), the CO_2 augmentation was more perceptible but remained low (Fig. 4). This could be explained by the occurrence of competition between the two bacterial species, possibly for carbon or for oxygen. However, when NDAB-grown *Methylobacterium* sp. strain JS178 was added to a 36-day-old filtered DN22 [^{14}C]RDX culture supernatant, the liberation of CO_2 increased from 31.1% ± 0.1% to 65.5% ± 2.4% after 26 additional days of incubation, indicating mineralization of NDAB. In these sequential cultures, the following carbon mass balance was calculated after 108 days of incubation: 66.9% ± 3.2% of the carbon evolved as CO_2, 1.2% ± 0.1% was incorporated into the biomass, and 26.3% ± 1.4% remained in the liquid phase. As mentioned above, the carbon remaining in the liquid phase is possibly methanol or unavailable formaldehyde; the latter is known to form covalent bonds or polymerize with a wide range of chemical species.

Regulation of NDAB degradation in strain JS178. To identify which nitrogen sources are utilized by isolate JS178, we incubated it in M-succinate with various nitrogen compounds. Nitrogen sources that supported growth were then tested for their ability to up-regulate the degradation of NDAB in resting-cell assays. The maximal degradation rate was defined as that obtained with resting JS178 pregrown in M-succinate and NDAB (2.52 μmol/liter/h ± 15%).

The growth of strain JS178 was strongly positive in M-suc-

cinate with $NaNO_2$, $(NH_4)_2SO_4$, or urea. However, none of the compounds induced NDAB degradation.

RDX and HMX did not support growth of JS178. As reported for *M. populi* (20), strain JS178 was able to transform RDX only in a rich medium, via a cometabolic process. For both strains, the detection of an RDX mono-nitroso derivative (MNX) and MEDINA indicates that an RDX reductive attack, rather than the known aerobic mechanism based on denitration, is occurring (data not shown) (20).

To test whether an amidohydrolase catalyzes the degradation of NDAB (which contains an amide linkage, R-NH-CHO [Fig. 1]), cells were grown on allantoin, an intermediate in the purine catabolic pathway known to require an amidohydrolase for its decomposition by *Escherichia coli* (7). The compound supported good growth of JS178. The NDAB degradation rate produced by allantoin was as high as that produced by NDAB.

The possibility that a purine degradative enzyme(s) might be involved in the degradation of NDAB is particularly interesting, because it may lead to a better understanding of the commensalism reported to occur between plants and methylobacteria (16). Allantoin is known to be a key intermediate in the assimilation, metabolism, transport, and storage of nitrogen in plants (Fig. 1) (17). The fact that allantoin stimulates the synthesis of an enzyme(s) that degrades NDAB suggests that the same enzymes are involved in both pathways. We are currently investigating the relationship between the enzymes involved in allantoin and NDAB degradation.

In conclusion, the biotic and abiotic cyclic nitramine ring cleavage product NDAB is degradable by the soil isolate *Methylobacterium* sp. strain JS178. NDAB served as a source of nitrogen for growth of the isolate and was converted to N_2O and CO_2. Our preliminary results indicate that one or several amidohydrolases, naturally involved in the purine catabolic pathway, might be involved in the degradation of the xenobiotic compound. The results reveal the potential for the sequential participation of rhodococci and methylobacteria to effect the complete degradation of RDX. *Methylobacterium* species might play a major role in phytoremediation of nitramine compounds, especially in view of the recent report that NDAB formed in leaves of hydroponic cultures of reed canary grass contaminated with RDX and exposed to simulated sunlight (15). The role of methylobacteria as endophytes would be particularly relevant.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology. Wiley & Sons, New York, N.Y.
3. **Bhushan, B., S. Trott, J. C. Spain, A. Halasz, L. Paquet, and J. Hawari.** 2003. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a rabbit liver cytochrome P450: insight into the mechanism of RDX biodegradation by *Rhodococcus* sp. strain DN22. Appl. Environ. Microbiol. **69:**1347–1351.
4. **Broder, M. F., and R. A. Westmoreland.** 1998. An estimate of soils contaminated with secondary explosives. Final report. SFIM-AEC-ET-CR-98002. U.S. Army Environmental Center, Kansas City, Mo.
5. **Coleman, N. V., D. R. Nelson, and T. Duxbury.** 1998. Aerobic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22. Soil Biol. Biochem. **30:**1159–1167.
6. **Coleman, N. V., J. C. Spain, and T. Duxbury.** 2002. Evidence that RDX biodegradation by *Rhodococcus* strain DN22 is plasmid-borne and involves a cytochrome P-450. J. Appl. Microbiol. **93:**463–472.
7. **Cusa, E., N. Obradors, L. Baldomà, J. Badìa, and J. Aguilar.** 1999. Genetic analysis of a chromosomal region containing genes required for assimilation of allantoin nitrogen and linked glyoxylate metabolism in *Escherichia coli.* J. Bacteriol. **181:**7479–7484.
8. **Fournier, D., A. Halasz, J. C. Spain, P. Fiurasek, and J. Hawari.** 2002. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. strain DN22. Appl. Environ. Microbiol. **68:**166–172.
9. **Fournier, D., A. Halasz, J. Spain, R. J. Spanggord, J. C. Bottaro, and J. Hawari.** 2004. Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal (NDAB) by *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **70:**1123–1128.
10. **Fournier, D., A. Halasz, S. Thiboutot, G. Ampleman, D. Manno, and J. Hawari.** 2004. Biodegradation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by *Phanerochaete chrysosporium*: new insight into the degradation pathway. Environ. Sci. Technol. **38:**4130–4133.
11. **Green, P. N.** 1992. The genus *Methylobacterium*, p. 2342–2349. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer-Verlag, New York, N.Y.
12. **Hawari, J.** 2000. Biodegradation of RDX and HMX: from basic research to field application, p. 277–310. *In* J. C. Spain, J. B. Hughes, and H.-J. Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. CRC Press, Boca Raton, Fla.
13. **Hawari, J., A. Halasz, C. Groom, S. Deschamps, L. Paquet, C. Beaulieu, and A. Corriveau.** 2002. Photodegradation of RDX in aqueous solution: a mechanistic probe for biodegradation with *Rhodococcus* sp. Environ. Sci. Technol. **36:**5117–5123.
14. **Jones, A. M., C. W. Greer, G. Ampleman, S. Thiboutot, J. Lavigne, and J. Hawari.** 1995. Biodegradability of selected highly energetic pollutants under aerobic conditions, p. 251–257. *In* R. E. Hinchee, D. B. Anderson, and R. E. Hoeppel (ed.), Bioremediation of recalcitrant organics. 1995 Third International In Situ Bioremediation Symposium. Battelle Press, Columbus, Ohio.
15. **Just, C. L., and J. L. Schnoor.** 2004. Phytophotolysis of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in leaves of reed canary grass. Environ. Sci. Technol. **38:**290–295.
16. **Omer, Z. S., R. Tombolini, and B. Gerhardson.** 2004. Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). FEMS Microbiol. Ecol. **47:**319–326.
17. **Schubert, K. R., and M. J. Boland.** 1990. The ureides, p. 197–282. *In* B. J. Miflin and P. J. Lea (ed.), The biochemistry of plants, a comprehensive treatise, vol. 16. Intermediary nitrogen metabolism. Academic Press, San Diego, Calif.
18. **Seth-Smith, H. M. B., S. J. Rosser, A. Basran, E. R. Travis, E. R. Dabbs, S. Nicklin, and N. C. Bruce.** 2002. Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus rhodochrous.* Appl. Environ. Microbiol. **68:**4764–4771.
19. **Van Aken, B., C. M. Peres, S. Lafferty Doty, J. M. Yoon, and J. L. Schnoor.** 2004. *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* × *nigra* DN34). Int. J. Syst. Evol. Microbiol. **54:**1191–1196.
20. **Van Aken, B., J. M. Yoon, and J. L. Schnoor.** 2004. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a photosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* × *nigra* DN34). Appl. Environ. Microbiol. **70:**508–517.