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Mannopine and Mannopinic Acid as Substrates for *Arthrobacter* sp. Strain MBA209 and *Pseudomonas putida* NA513

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The characteristics of mannopine and mannopinic acid utilization by *Agrobacterium tumefaciens* B6S3, *Arthrobacter* sp. strain MBA209, and *Pseudomonas putida* NA513 were studied. Strain B6S3 utilized the four mannityl opines, mannopine, mannopinic acid, agropine, and agropinic acid. It also utilized several mannityl opine analogs, which were modified in either the sugar or the amino acid moiety. It utilized mannopine more rapidly after preincubation on mannopine, mannopinic acid, or glutamine than after pregrowth on glucose, mannose, or mannitol. Strains MBA209 and NA513 utilized mannopine and mannopinic acid, but not the other two mannityl opines. They utilized few mannityl opine analogs, sometimes because of failure to utilize the products of initial cleavage of the analog. Utilization of mannopine and mannopinic acid by strain NA513 was strictly dependent on prior growth on these substrates. A spontaneous regulatory variant of strain NA513 remained unable to utilize most of the mannityl opine analogs. Glutamine, mannose, and several analogs had no inhibitory effect on [^{14}C]mannopine utilization by strain NA513.

The soil microbe *Agrobacterium tumefaciens* causes crown gall disease in a wide range of dicotyledonous plants. Crown gall tumors contain low-molecular-weight metabolites called opines which are not found in non-tumorous plants. *A. tumefaciens* incites tumors by transferring part of the tumor-inducing (Ti) plasmid into the host plant cell. The transferred DNA of bacterial origin, or T-DNA, becomes integrated into the plant genome (6, 23, 27, 36) where it is expressed, determining tumor development and the synthesis of one or more opines (5, 16, 21, 23, 29). *Agrobacterium rhizogenes* causes the related hairy root disease by a similar mechanism involving a root-inducing (Ri) plasmid (10, 51). More than a dozen opines have been isolated (9, 12–15, 20, 22, 41, 43–45). Agrobacteria, and the Ti plasmids they carry, are classified according to the types of opines present in the tumors they incite (48). Opines are catabolized by the inciting agrobacteria at the direction of genes localized on a nontransferred region of the Ti plasmids. A direct correspondence exists between the biosynthetic activity expressed by crown gall tumors and the catabolic potential of the inciting agrobacteria (38). Opines can be used as the sole carbon source and, in most cases, also as the sole nitrogen source. Opine catabolism by agrobacteria has been found to be inducible in all instances which have been investigated (11, 30, 47).

Opine catabolism is also a property of various types of microorganisms not belonging in the genus *Agrobacterium* (1, 2, 7, 8, 31, 32, 40, 49). Pseudomonads and coryneform bacteria utilizing opines of the imino diacid type, such as octopine, nopaline, succinamopine, and leucinopine, are readily recovered from soil and plant samples (2, 3, 7, 39, 49). Octopine specifically induces an octopine permease activity in *Pseudomonas putida* 203 (4). A recent study concluded that octopine was a substrate of poor selectivity for agrobacteria, its use as the sole carbon source in selective media resulting in the exclusive recovery of pseudomonads. In contrast, the mannityl opine mannopine (MOP) was utilized predominantly by agrobacteria and coryneform bacteria. Only a few MOP-utilizing pseudomonads were recovered, and these were indistinguishable from each other on the basis of the determinative tests used in the study. The type pseudomonad isolate was designated as *Pseudomonas putida* NA513 (35).

Four mannityl opines have been described. The chemical constituents of MOP and mannopinic acid (MOA) are glutamine and glutamic acid, respectively, and mannose. Two other opines are derived from MOP: formation of a lactam yields agropinic acid (AGA), while formation of a lactone yields agropine. An extensive series of analogs of the mannityl opines has been synthesized that differ from the natural opines in either the amino acid or the sugar moiety (11). The objective of the present study was to investigate the utilization of mannityl opines and some of their analogs by *P. putida* NA513 and the coryneform bacterium *Arthrobacter* sp. strain MBA209. The catabolic character expressed by these strains was also compared with that of *A. tumefaciens* B6S3, which utilizes all four mannityl opines.

MATERIALS AND METHODS

Bacteria and culture conditions. The sources of bacterial strains used in this study are listed in Table 1. *A. tumefaciens* B6S3 is a derivative of strain B6 (25). *P. putida* NA513 and *Arthrobacter* sp. strain MBA209 were isolated on selective medium with MOP as the sole carbon source (35) and identified by the American Type Culture Collection. Bacteria were maintained on solid minimal medium based on AT salts (24), which contained the following, per liter: KH_2PO_4, 10.9 g; $MgSO_4 \cdot 7H_2O$, 0.16 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $CaCl_2 \cdot 2H_2O$, 0.011 g; and $MnCl_2 \cdot 4H_2O$, 0.002 g; the pH of the solution was adjusted to 7.0 by adding KOH. In addition to AT salts, solid minimal medium contained 15 g of Noble agar (Difco) per liter, 1 mg of $(NH_4)_2SO_4$ per ml, and 1 mg of opine or analog per ml as the sole carbon source. Liquid cultures were incubated at 25°C with shaking. Generation time of bacteria in liquid culture was determined on exponentially growing cells by measuring optical density

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Agrobacterium tumefaciens B6S3	Contains octopine-type pTiB6S3	J. Tempé
Pseudomonas putida NA513	Soil isolate	35
Pseudomonas putida NA513B	Spontaneous variant of NA513, isolated on MAN-HIS	This study
Arthrobacter sp. strain MBA209	Potato isolate	35
Plasmids		
pE43	Contains *Hin*dIII fragment C from TR-DNA of pTiB6-806, cloned in vector pBR325	28
pYDH208	Contains *Hin*dIII fragments 2, 9a, 18a, and 25a of pTi15955, cloned in vector pCP13/B	19

(OD) at 600 nm every 2 h, using a Novaspec spectrophotometer (LKB). Exponential phase generally occurred over the OD range 0.3 to 0.9.

Preparation of opines and analogs. Opines and opine analogs (see Fig. 1 and 2 for structures) were prepared by published procedures (11). The following abbreviated names of *N*-1-deoxyalditol analogs are used throughout: N^{α}-1-(1-deoxy-D-glucityl)-L-glutamine, GLC-GLN; N^{α}-1-(1-deoxy-D-galactityl)-L-glutamine, GAL-GLN; N^{α}-1-(1-deoxy-L-rhamnityl)-L-glutamine, RHA-GLN; N^{α}-1-(1,2-dideoxy-D-mannityl)-L-glutamine, deoxy-MOP; N^{α}-1-(1-deoxy-D-ribityl)-L-glutamine, RIB-GLN; N^{α}-1-(1-deoxy-L-arabinityl)-L-glutamine, ARA-GLN; N^{α}-1-(1-deoxy-D-xylityl)-L-glutamine, XYL-GLN; N^{α}-ethyl-L-glutamine, ET-GLN; N^{α}-methyl-L-glutamine, ME-GLN; *N*-1-(1-deoxy-D-galactityl)-L-glutamic acid, GAL-GLU; *N*-1-(1,2-dideoxy-D-mannityl)-L-glutamic acid, deoxy-MOA; *N*-1-(1-deoxy-D-xylityl)-L-glutamic acid, XYL-GLU; *N*-1-(1-deoxy-D-erythrityl)-L-glutamic acid, ERY-GLU; *N*-1-(*R*-2,3-dihydroxypropyl)-L-glutamic acid, DHP-GLU; *N*-methyl-L-glutamic acid, ME-GLU. The following abbreviations are used for *N*-1-(1-deoxy-D-mannityl) derivatives of various amino acids: L-asparagine, nor-MOP; L-aspartic acid, nor-MOA; glycine, MAN-GLY; L-serine, MAN-SER; L-arginine, MAN-ARG; L-citrulline, MAN-CIT; L-histidine, MAN-HIS; L-proline, MAN-PRO; α-aminoisobutyric acid, MAN-AIB. The analog 1-amino-1-deoxy-D-mannitol, abbreviated MAN-NH_2, was similarly prepared by reduction of D-mannose with sodium cyanoborohydride in the presence of a large excess of ammonia, and N^{α}-methylman-

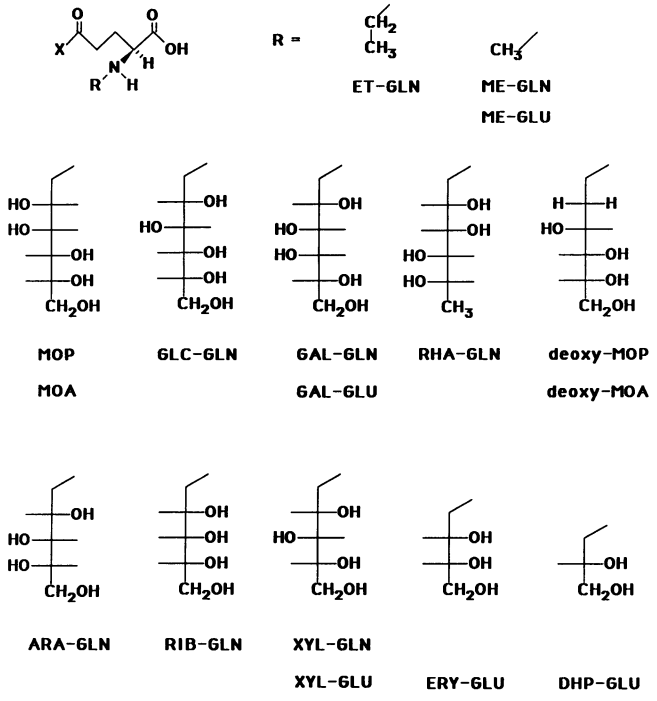

FIG. 1. MOP, MOA, and deoxyalditol analogs of these mannityl opines. X = NH_2 for MOP and MOP analogs; X = OH for MOA and MOA analogs.

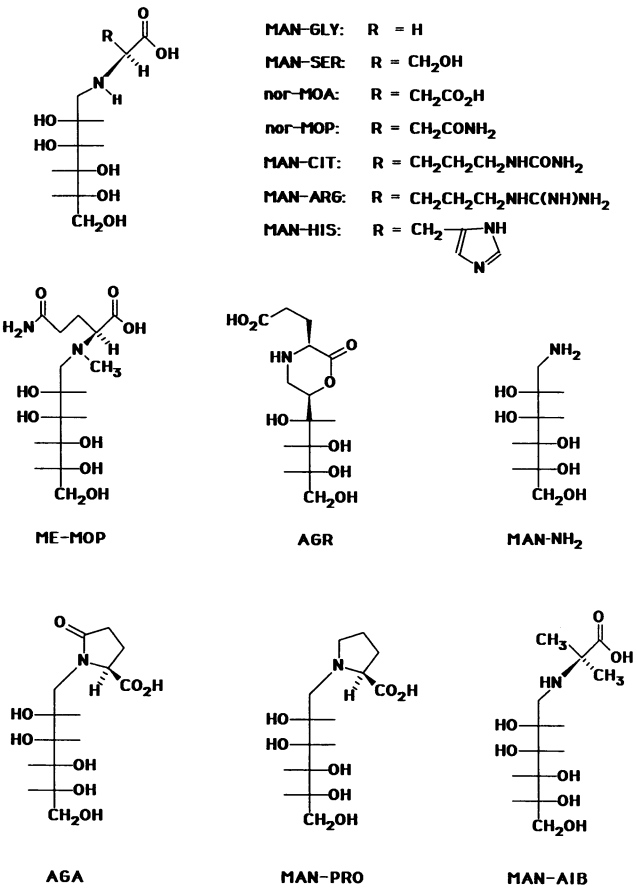

FIG. 2. AGR, AGA, and amino acid analogs of MOP and MOA.

nopine, abbreviated ME-MOP, was prepared by reductive methylation of MOP in the presence of a large excess of formaldehyde. Opine and analog stock solutions (40 mg/ml) were neutralized, filter sterilized, and stored at −20°C.

[^{14}C]MOP and [^{14}C]MOA (specific activity, 270 μCi/μmol) were prepared from uniformly labeled mannose by published procedures (11). Just before use, [^{14}C]MOP was freed of AGA by absorption onto Dowex 50 ion-exchange resin and elution with dilute ammonia. The eluate, after evaporation of ammonia, was analyzed by paper electrophoresis at pH 1.8 (11). Greater than 95% of the radioactivity was in [^{14}C]MOP and <5% was in [^{14}C]AGA. Electrophoretic analysis of the stock [^{14}C]MOA solution indicated that there was no [^{14}C]AGA present.

Detection of opine and opine analog in culture supernatant fluids. Bacterial inoculum was prepared in medium containing AT salts, 1 mg of ammonium sulfate per ml, and 1 mg of glucose per ml. Exponentially growing cells were centrifuged, washed four times in AT salts, and resuspended in AT salts at an OD at 600 nm of 0.5. A volume of 0.1 ml of suspension was transferred to 2 ml of medium containing AT salts, 1 mg of ammonium sulfate per ml, and, unless stated otherwise, 1 mg of opine or opine analog per ml. At 24, 48, 72, 96, 168, and 240 h, 0.2-ml samples were centrifuged and 20 μl of supernatant fluid was subjected to electrophoresis on 3MM paper (Whatman) at pH 1.8. Octopine and nopaline were detected with phenanthrenequinone (37). Agropine, MOP, MOA, and mannityl opine analogs were detected with the silver nitrate reagent (11).

Detection of intermediates and end products of catabolism. Amino acids were detected in liquid bacterial cultures (see preceding section) by subjecting 20 μl of culture supernatant to paper electrophoresis at pH 1.8, 4.0, or 9.6 (11) and treating the papers with ninhydrin. Sugars were detected by subjecting 20 μl of culture supernatant fluid to chromatography on 3MM paper alongside synthetic standards. The chromatograms were developed with 1-butanol–acetic acid–water (12:3:5), and the sugars were revealed by treating with silver nitrate (11). When putative sugar intermediates were detected, they were confirmed as aldoses by treating a second chromatogram with aniline phosphate (11).

To test whether bacteria grown in the presence of MOP excrete an enzyme capable of cleaving this opine, liquid minimal medium containing AT salts, ammonium sulfate, and 1 mg of MOP per ml was inoculated, and the culture was incubated until it reached an OD of 0.5. Exponentially growing cells were removed by centrifugation before all MOP had disappeared, and the supernatant was filter sterilized. It was then incubated at 28°C, and 500-μl samples were taken at 0, 24, and 48 h. The samples were concentrated to a final volume of 20 μl under vacuum, and 5 μl of concentrated sample was subjected to paper electrophoresis at pH 9.6. The papers were stained with ninhydrin or silver nitrate.

Studies with radiolabeled opines. Bacteria were prepared in 3 ml of pregrowth medium containing AT salts, 1 mg of ammonium sulfate per ml, and 1 mg of carbon source per ml. When the culture reached an OD at 600 nm of 0.5, exponentially growing cells were collected by centrifugation, washed four times with AT salts and resuspended in 3 ml of AT salts. The bacterial suspension was adjusted to an OD at 600 nm of 0.2, and 200 μl of the resulting inoculum was added to 1.5 ml of assay medium containing AT salts, 1 mg of ammonium sulfate per ml, and 13,500 cpm of [^{14}C]MOA (18.8 nM, final concentration) or 12,000 cpm of [^{14}C]MOP (16.6 nM, final concentration). Bacteria were incubated at 25°C with shaking. At intervals, samples were centrifuged and 100 μl of supernatant fluid was assayed by liquid scintillation counting. For competitive inhibition experiments, the test inhibitor was added to assay medium at 0.3 to 0.4 mM (0.1 mg/ml) unless stated otherwise. MAN-NH_2 was tested as an inhibitor at 0.55 mM (0.1 mg/ml). The presence of the tested compound at the end of the experiment was verified by paper electrophoresis or chromatography (see above).

Incorporation of the ^{14}C label into the macromolecular fraction was monitored by trichloroacetic acid precipitation (26).

All experiments involving radiolabeled substrates were done at least three times.

Preparation of DNA probes. The probe for the genes of MOP synthesis consisted of the 0.8-kb *Hin*dIII fragment C from the TR-DNA of pTiB6-806 cloned in plasmid pBR325 (Table 1). This fragment contains part of the 2′ gene encoding the first step in MOP synthesis (28). The MOP catabolic gene probe was the 5-kb *Hin*dIII fragment 9a from pTi15955 cloned in pYDH208 (Table 1). This fragment is essential for MOP catabolism (19). Probes were eluted twice from 0.5% low-melting agarose gel (Bethesda Research Laboratory), as described by Maniatis et al. (33). They were labeled by nick translation in the presence of [^{32}P]dCTP (using a Bethesda Research Laboratory nick translation kit) to a specific activity of 8×10^7 cpm per μg of DNA.

Isolation of total DNA and Southern hybridization. Total DNA was isolated as described by Meade et al. (34), except that 10 mg of sodium dodecyl sulfate (SDS) per ml was used to lyse the cells in place of Sarkosyl and pronase. The DNA (5 μg) was digested with the restriction enzyme *Eco*RI (Pharmacia). Agarose gel electrophoresis and Southern blotting were performed as described by Maniatis et al. (33). DNA-DNA filter hybridization was done at 42°C in the presence of 50% (vol/vol) formamide (33). The probes were used at a concentration of 100 ng/ml of hybridization buffer. Two 30-min washes with 300 mM sodium chloride–30 mM sodium citrate buffer, containing 0.1% (wt/vol) SDS, were performed at 22°C. The filters were subjected to autoradiography using X-Omat AR film (Kodak) and two intensifying screens.

RESULTS

Utilization of amino acids, sugars, and mannityl opines. *A. tumefaciens* B6S3, *Arthrobacter* sp. strain MBA209, and *P. putida* NA513 were tested for growth on several sugars and amino acids as the sole carbon source. Strain B6S3 utilized most of the 16 substrates tested, while strains MBA209 and NA513 exhibited a greater potential for amino acid than for sugar utilization (Table 2). The generation time of the three species of bacteria on MOP as well as on the two components of this mannityl opine, mannose and glutamine, was generally of the same order as that measured on glucose (Table 3). *A. tumefaciens* B6S3 utilized the four mannityl opines, whereas *Arthrobacter* sp. strain MBA209 and *P. putida* NA513 utilized MOP and MOA, but not agropine or AGA (Table 4). Disappearance of mannityl opine from inoculated media was always accompanied by an increase in turbidity. No AGA was detected in uninoculated or inoculated medium containing MOP or MOA during the course of these experiments.

The stability of MOP was tested in sterile culture filtrates of strains MBA209 and NA513 which had been grown on MOP. No amino acid or sugar was detectable after allowing such sterile solutions of MOP to stand for 48 h. Traces of AGA were detected, as they were in an uninoculated con-

TABLE 2. Ability of bacteria to grow on amino acids and sugars as the sole carbon source[a]

Carbon source	Growth of strain:		
	B6S3	MBA209	NA513
L-Arabinose	+	+	−
D-Erythrose	−	−	−
D-Galactose	+	+	−
L-Rhamnose	+	−	−
D-Ribose	+	−	+
D-Xylose	+	−	+
L-Alanine	+	+	+
L-Arginine	+	+	+
L-Asparagine	+	+	+
L-Aspartate	+	+	+
L-Citrulline	+	−	+
L-Glutamate	+	+	+
Glycine	−	+	+
L-Histidine	+	+	+
L-Proline	+	+	+
L-Serine	+	−	+

[a] Bacteria were incubated in minimal medium containing AT salts, 1 mg of ammonium sulfate per ml, and 1 mg of carbon source per ml. +, OD at 600 nm of >0.5 after 7 days; −, absence of growth after 7 days.

trol. This indicated that these two bacteria did not produce extracellular enzymes responsible for MOP breakdown.

Utilization of MOP and MOA analogs. Two types of analogs were used in catabolic studies. Some analogs, the deoxyalditiyl analogs, were prepared by varying the deoxymannityl portion of MOP or MOA (Fig. 1). For seven analogs of MOP, the *N*-1-deoxymannityl group was replaced with deoxyalditiyl groups having different stereochemistries and chain lengths, while in ET-GLN the deoxymannityl group was replaced with an ethyl group. For five analogs of MOA the deoxyalditiyl chain length was varied from 1 through 6 carbon atoms. The analogs deoxy-MOP and deoxy-MOA lacked the C-2 hydroxyl (Fig. 1). In 11 other analogs, the amino acid analogs, the deoxymannityl portion was held constant and the L-amino acid moiety was systematically varied (Fig. 2). Most of the sugar and amino acid components of the mannityl opine analogs were assayed as the sole carbon source in nutritional tests (Table 2).

A. tumefaciens B6S3 utilized all of the 14 deoxyalditiyl MOP and MOA analogs tested. Strain B6S3 also utilized four of the eight amino acid analogs tested. In contrast, *Arthrobacter* sp. strain MBA209 exhibited specificity for the deoxymannityl portion of mannityl opines, utilizing only

TABLE 3. Generation time of bacteria grown on MOP and related substrates[a]

Carbon source	Generation time (h) of strain:			
	B6S3	MBA209	NA513	NA513B
Glucose	4.5	7.5	6.4	6.0
Mannose	6.0	11.2	6.8	6.0
Glutamine	6.0	7.1	3.8	3.8
Glutamate	5.6	8.3	4.0	3.6
Histidine	NT	NT	4.0	3.8
MOP	9.0	7.1	6.8	6.8
MOA	8.0	6.7	10.5	10.1
MAN-HIS	NT	NT	NG	6.4

[a] Bacteria were incubated in minimal medium containing AT salts, 1 mg of ammonium sulfate per ml, and 1 mg of carbon source per ml. NT, Not tested; NG, no growth after 3 days.

TABLE 4. Time required for complete utilization of opines by bacteria

Bacterial strain	Time (h) required for complete utilization of:					
	OCT	NOP	MOP	MOA	AGR	AGA
A. tumefaciens B6S3	24	—[a]	24	24	48	24
P. putida NA513	—	—	24	24	—	—
Arthrobacter sp. strain MBA209	—	—	72	120	—	—

[a] —, Opine not utilized within 240 h. OCT, Octopine; NOP, nopaline; AGR, agropine.

GLC-GLN in less than 24 h, while all of the other deoxyalditiyl analogs were still detected in the culture supernatant after 168 h of incubation. Among the amino acid analogs tested, only two, MAN-SER and MAN-ARG, were utilized by this bacterium. *P. putida* NA513 utilized none of the six deoxyalditiyl analogs of MOA and only one of the eight analogs of MOP tested, RIB-GLN, which was detected up to 168 h after inoculation, but was completely utilized at 240 h. Results for strain NA513 on the amino acid analogs were similar to those described above for strain B6S3 (Table 5).

The complete utilization of an analog of MOP or MOA, which was revealed by the disappearance of this substrate from the culture supernatant fluid, was always correlated with an increase in turbidity of the culture. Conversely, no increase in culture turbidity was observed in those cases in which an analog did not disappear from the medium during a 240-h incubation period.

Isolation of a spontaneous variant of NA513 with the capacity for rapid growth on MAN-HIS and further studies on the utilization of analogs. Strain NA513 required 168 h to utilize completely the mannityl opine analog MAN-HIS. Complete utilization at this time may have been due to slow growth of all of the cells or else absence of growth of most cells accompanied by the selection of MAN-HIS utilizers. To isolate a spontaneous derivative of NA513 with the capacity to utilize MAN-HIS rapidly, 10^8 NA513 cells which had been grown on AT salts with ammonium sulfate and glucose were plated on solid minimal medium with 2 mg of MAN-HIS per ml as the sole carbon source. Ten colonies which appeared after 2 weeks were purified on MAN-HIS. The resulting strains differed in the stability of their variant character, and the most stable of them, strain NA513B, was selected for further studies. The variant character of NA513B was confirmed by showing that this strain grew readily on MAN-HIS (Table 3) and required only 48 h to utilize this substrate completely (Table 5). However, this variant character was unstable, since 97% of the cells had lost the ability to grow on MAN-HIS after five successive transfers on liquid minimal medium containing glucose as the carbon source. Compared with the parent strain NA513, the spontaneous variant exhibited an enhanced capacity to utilize nor-MOA and nor-MOP, but remained unable to utilize 13 other deoxyalditiyl or amino acid analogs tested (Table 5).

The ability of strain NA513 to utilize mannityl opine analogs in the presence of MOP was also examined. The results were similar to those described above for NA513B (Table 5).

Excretion of intermediates and end products of catabolism. Upon incubation on some of the analogs, strain B6S3 released into the culture medium intermediate aldoses which would be derived by hydrogen abstraction from C-1 of the

TABLE 5. Time required for complete utilization of mannityl opine analogs by bacteria[a]

Type and designation of analog	Time (h) required in the absence of MOP by strain:				Time (h) required in the presence of MOP by strain NA513
	B6S3	MBA209	NA513	NA513B	
Deoxyalditol analogs of MOP					
GLC-GLN	48	24	—	NT	NT
GAL-GLN	96	—	—	NT	NT
RHA-GLN	72	—	—	—	—
Deoxy-MOP	96	—	—	—	—
ARA-GLN	72	—	—	—	—
RIB-GLN	72	—	240	NT	NT
XYL-GLN	24	—	—	—	—
ET-GLN	48	240	—	NT	NT
Deoxyalditol analogs of MOA					
GAL-GLU	48	—	—	—	—
Deoxy-MOA	24	—	—	—	—
XYL-GLU	24	—	—	—	—
ERY-GLU	24	—	—	—	—
DHP-GLU	24	—	—	NT	NT
ME-GLU	96	—	—	NT	NT
Amino acid analogs					
MAN-SER	48	72	48	NT	NT
MAN-AIB	—	—	—	—	—
MAN-CIT	72	—	48	NT	NT
MAN-ARG	—	240	—	—	—
MAN-PRO	—	—	—	—	—
MAN-HIS	72	—	168	48	48
nor-MOA	—	—	—	240	240
nor-MOP	NT	NT	72	24	24
MAN-GLY	96	—	96	NT	NT
MAN-NH_2	NT	NT	—	—	—
ME-MOP	NT	NT	—	—	—

[a] In the absence of MOP, analogs were present in the assay medium at an initial concentration of 1 mg/ml. When in combination with 0.5 mg of MOP per ml, analogs were added to the assay medium at an initial concentration of 0.5 mg/ml. —, Analog not utilized within 240 h. NT, Not tested.

alditol side chain of the substrate. This bacterium accumulated 2-deoxy-D-mannose (= 2-deoxy-D-glucose) and D-erythrose during growth on deoxy-MOP and ERY-GLU, respectively. Consistent with this, no growth of strain B6S3 on D-erythrose was observed within 168 h (Table 2). Accumulation of 2-deoxy-D-mannose and D-erythrose during catabolism of deoxy-MOP and ERY-GLU has been noted previously for a strain carrying pTiB6-806 (11). Other cases of extracellular accumulation of intermediates during catabolism of mannityl opines or analogs have been reported (11, 18).

Arthrobacter sp. strain MBA209 liberated glutamic acid into the medium during growth on MOP, MOA, and ET-GLN and this amino acid remained detectable until the end of the experiment. The transient accumulation of glutamine was also noticed in some experiments. Upon incubation on XYL-GLN, strain MBA209 liberated D-xylose, which could be detected from 96 h on. However, the culture did not show an increase in turbidity, and no reduction in the concentration of the substrate XYL-GLN could be noticed by electrophoretic analysis of culture supernatant fluid. Thus, the utilization of glutamate by strain MBA209, which was observed when this amino acid was present as the sole carbon source (Table 2), was suppressed in the presence of a mannityl opine or a mannityl opine analog. This suppression was observed even with the MOP analog XYL-GLN, which upon cleavage generated the nonutilizable sugar D-xylose.

Factors influencing the bacterial ability to cause disappearance of radiolabeled MOP and MOA. In some experiments, the utilization of MOP and MOA by bacteria was measured by monitoring the disappearance of radiolabeled substrate from the culture medium in the presence of bacteria subjected to various pregrowth conditions. Most of the [^{14}C]MOP and [^{14}C]MOA disappeared from a culture of strain B6S3 within 30 min when the cells had been pregrown on MOP or MOA, while disappearance of the radiolabeled substrates was much slower after pregrowth on glucose (Fig. 3A and C). Cells of NA513 which had been pregrown on MOP utilized 70% of the [^{14}C]MOP offered within 1 h and about 50% of the [^{14}C]MOA in a 2-h period. Following pregrowth on MOA, NA513 cells utilized [^{14}C]MOP, but no detectable utilization of [^{14}C]MOA was observed. NA513 cells pregrown on glucose showed no utilization of [^{14}C]MOP or [^{14}C]MOA (Fig. 3B and D).

The variant NA513B, which had spontaneously acquired an enhanced capacity to utilize MAN-HIS (see above), demonstrated a rapid utilization of [^{14}C]MOP whether pregrown on glucose, MOP, or MAN-HIS (Fig. 4).

MOA at 0.1 mg/ml (0.3 mM) did not inhibit the utilization of [^{14}C]MOP by cells of B6S3 (results not shown) and NA513 (Fig. 5A) which had been pregrown on MOP. In the case of strain NA513, the ability to utilize [^{14}C]MOP was also tested in the presence of higher concentrations of MOA, and some inhibition was noted under these circumstances (Fig. 5A). MOP at 0.1 mg/ml (0.3 mM) completely blocked the utilization of [^{14}C]MOA by cells of B6S3 (results not shown) and NA513 (Fig. 5B).

The effect of sodium azide on the disappearance of [^{14}C]MOP was examined to determine whether metabolically functional bacteria were required. Sodium azide at 10 mM has been used previously to inhibit energy-dependent uptake of opines by *A. tumefaciens* (50). Cells of B6S3 and

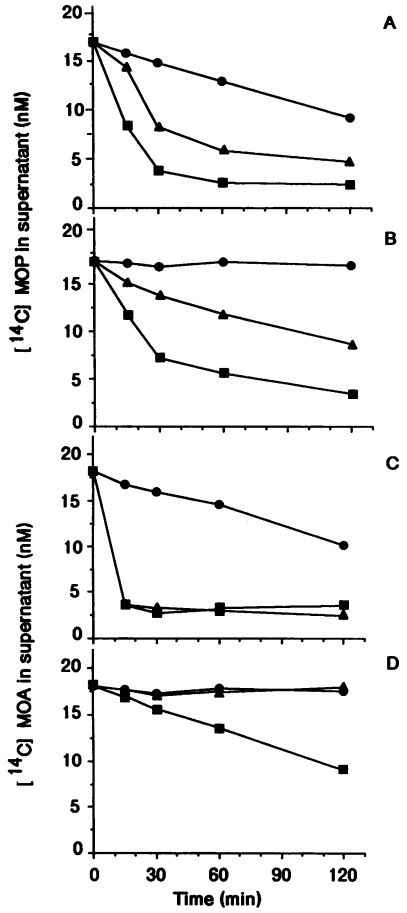

FIG. 3. Utilization of [^{14}C]MOP and [^{14}C]MOA by *A. tumefaciens* B6S3 (A and C, respectively) and *P. putida* NA513 (B and D, respectively). Bacteria were pregrown on 1-mg/ml glucose (●), MOP (■), or MOA (▲) as the sole carbon source.

NA513 were pregrown on MOP, harvested, washed as described in Materials and Methods, and resuspended in assay medium without radiolabeled substrate but with or without 10 mM sodium azide. [^{14}C]MOP was added after 1 h and was found to disappear from culture medium of B6S3 and NA513 only in the absence of azide (data not shown).

Disappearance of [^{14}C]MOP from the medium was accompanied by incorporation of some of the label into the trichloroacetic acid-precipitable macromolecular material, although the actual fraction of label incorporated varied between experiments. In 40 min of incubation on [^{14}C]MOP, cells of B6S3 pregrown on MOP utilized 60% of the radioactivity initially present in the medium and incorporated 16% of this utilized radioactivity into the trichloroacetic acid-precipitable fraction. Following pregrowth on glucose, B6S3 cells utilized 15% of the [^{14}C]MOP offered and incorporated 3% of this utilized substrate in the trichloroacetic acid precipitate. Cells of NA513 pregrown on MOP utilized 50% of the [^{14}C]MOP in 45 min and exhibited a 20% level of incorporation. Neither utilization nor incorporation of the [^{14}C]MOP was observed for NA513 cells pregrown on glucose.

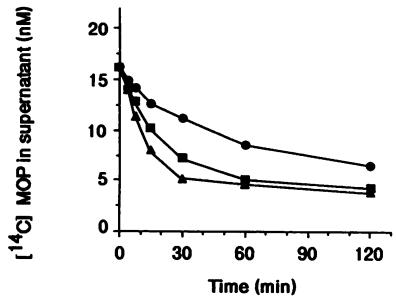

FIG. 4. Utilization of [^{14}C]MOP by *P. putida* NA513B. Bacteria were pregrown on 1-mg/ml glucose (●), MOP (■), or MAN-HIS (▲) as the sole carbon source.

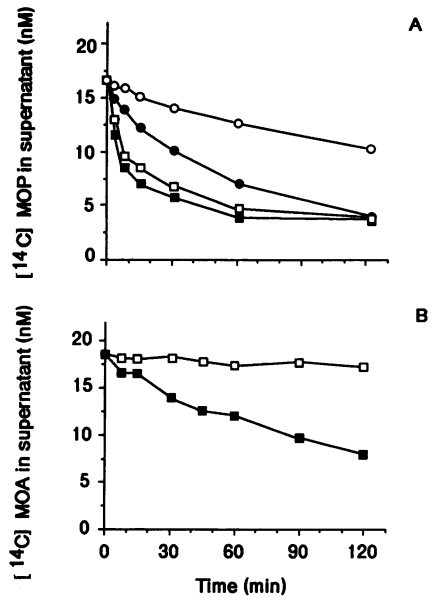

FIG. 5. Utilization of [^{14}C]MOP and [^{14}C]MOA by *P. putida* NA513. (A) Bacteria were pregrown on 3 mM (1 mg/ml) MOP as the sole carbon source and assayed for utilization of [^{14}C]MOP either alone (■) or in the presence of unlabeled MOA at 0.3 (□), 1.6 (●), or 8 (○) mM. (B) Bacteria were pregrown on 3 mM MOP as the sole carbon source and assayed for utilization of [^{14}C]MOA either alone (■) or in the presence of unlabeled MOP at 0.3 mM (□).

Effect of pregrowth on mannityl opine components, analogs, and related compounds on [^{14}C]MOP utilization. Following pregrowth on MOP and glutamine, strain B6S3 utilized [^{14}C]MOP within 60 min (Fig. 6A). Significant, but incomplete, utilization of [^{14}C]MOP by glucose-pregrown cells occurred over a 120-min time period, in accordance with previous results (Fig. 3A). The effect of mannose was similar to that of glucose. Almost no utilization was obtained with mannitol-pregrown cells, which is in agreement with previously published results on pTiB6-806 (11). The utilization of [^{14}C]MOP by strain NA513 was noted after pregrowth on MOP or nor-MOP, but not glutamine, glucose, or mannose (Fig. 6B). Strain NA513 does not utilize mannitol, and hence the effect of pregrowth on this compound could not be tested.

Mannityl opine components and analogs as competitors of MOP utilization. The utilization of [^{14}C]MOP by strain NA513 was not affected by the presence of 0.7 mM glutamine or 0.6 mM mannose. None of eight deoxyaldityl analogs tested at a 0.3 mM concentration, RHA-GLN,

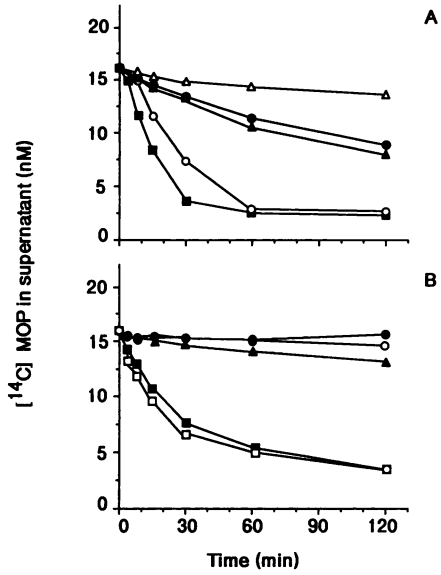

FIG. 6. Utilization of [^{14}C]MOP by *A. tumefaciens* B6S3 (A) and *P. putida* NA513 (B). Bacteria were pregrown on 1-mg/ml glucose (●), mannose (▲), mannitol (△), glutamine (○), MOP (■), or nor-MOP (□) as the sole carbon source.

deoxy-MOP, ARA-GLN, XYL-GLN, GAL-GLU, deoxy-MOA, XYL-GLU, and ERY-GLU, nor 0.6 mM ME-GLN showed activity as an inhibitor of [^{14}C]MOP utilization by NA513 cells which had been pregrown on MOP (data not shown). Similarly, four amino acid analogs (MAN-ARG, MAN-PRO, MAN-HIS, and nor-MOA) at 0.3 mM and MAN-NH_2 at 0.6 mM did not act as competitors (data not shown). However, inhibition of [^{14}C]MOP utilization by NA513 was observed in the presence of 0.3 to 8 mM nor-MOP (Fig. 7) and MAN-AIB (results not shown).

Hybridization analysis with probes derived from Ti plasmid-encoded genes of MOP metabolism. Total DNA from strain B6S3 showed homology to DNA probes derived from Ti plasmid-encoded genes of MOP synthesis and catabolism. In contrast, no hybridization was detected to total DNA from strains MBA209 and NA513 (data not shown).

DISCUSSION

Early studies on opine biosynthesis and catabolism have introduced the concept of specificity (38), and indeed this notion has become central to our understanding of crown gall disease. Most opines are found only in genetically transformed plant tissue (46) and are believed to be catabolized preferentially, if not solely, by the transforming bacteria (48). Research on *Agrobacterium* biology suggests that this postulated specificity of catabolism may, in fact, not be absolute. Various types of Ti and Ri plasmids have been described, some of which confer on their respective hosts the capacity to utilize the same opines. Hence, agrobacteria harboring different Ti or Ri plasmids could in principle compete with one another for a given opine substrate. Other studies (see introduction) have expanded this hypothesis of competition for opines to other bacterial groups or attempted to observe this competition in extracts of crown gall tumors (42).

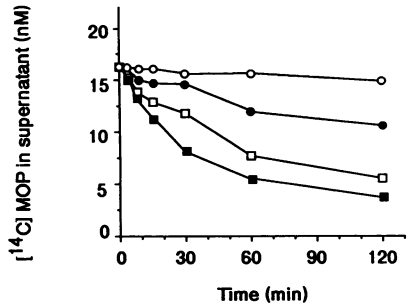

FIG. 7. Utilization of [^{14}C]MOP by *P. putida* NA513. Bacteria were pregrown on 3 mM MOP and tested for utilization of [^{14}C]MOP either alone (■) or in the presence of unlabeled nor-MOP at 0.3 (□), 1.7 (●), or 8 (○) mM.

The present study compares three bacteria belonging in different genera for their ability to utilize mannityl opines and some of their analogs. Hybridization analysis of total DNA from *A. tumefaciens* B6S3, *Arthrobacter* sp. strain MBA209, and *P. putida* NA513 shows that the genes of mannityl opine catabolism from strains MBA209 and NA513 are not closely related to Ti plasmid-encoded genes involved in biosynthesis or catabolism of MOP. Catabolic properties of these three bacteria were studied by monitoring disappearance of radiolabeled or unlabeled test substrates from culture media. Several lines of observations suggest that the data thus obtained reflect bacterial ability to utilize the test substrates for growth, through processes of uptake and catabolism. Disappearance of the test substrate and increase in turbidity of the culture remain strictly correlated. Except for a few bacterium-test substrate combinations (see Results), extracellular accumulation of catabolic intermediates is not detected by using silver nitrate or ninhydrin. The only plausible transformation products of test substrates which would not be detected by analysis of culture supernatant fluids with these reagents are CO_2, resulting from total respiration, and intermediates incorporated into cellular material. Indeed, incorporation of radioactivity derived from [^{14}C]MOP uniformly labeled in the deoxymannityl moiety is demonstrated for strains B6S3 and NA513. Finally, disappearance of [^{14}C]MOP in the presence of B6S3 or NA513 cells requires metabolic energy as shown by the effect of added sodium azide.

Enzymes of mannityl opine catabolism encoded by several Ti or Ri plasmids, including pTiB6-806, were shown previously to harbor little specificity at the substrate affinity level. Rather, specificity of the mannityl opine catabolic systems, which exists to a variable extent in different agrobacteria, depends on regulatory mechanisms (11). The *Agrobacterium* strain studied here carries pTiB6S3, a plasmid related to pTiB6-806 which had been found before to confer a broad catabolic potential on its host (11). Accordingly, strain B6S3 utilizes the four mannityl opines (Table 4) as well as several deoxyaldityl and amino acid analogs (Table 5). Thus, it represents a valuable positive control in the experiments described here.

In *Agrobacterium* spp., distinct catabolic pathways were postulated for MOP and AGA, while MOA can be degraded through either pathway (11, 18). An agropine cyclase activity may be specifically required in *Agrobacterium* spp. for agropine catabolism (17). Thus, the phenotype of strains MBA209 and NA513 with respect to mannityl opine utilization (Table 4) corresponds to the agrobacterial expression of the MOP catabolic pathway alone. For strain NA513, this hypothesis is supported by observations on the relationship between the utilization of MOP and that of MOA. Strain

NA513 grows slightly faster on MOP than on MOA (Table 3). Cells pregrown on MOP actively utilize [^{14}C]MOP and [^{14}C]MOA, while cells pregrown on MOA utilize [^{14}C]MOP, but not [^{14}C]MOA, over a 120-min time period (Fig. 3B and D). Pregrowth on the two components of MOP, mannose and glutamine, does not result in [^{14}C]MOP utilization by strain NA513 (Fig. 6B). MOP at 0.3 mM completely inhibits MOA utilization, while higher concentrations of MOA are required for a partial effect on MOP utilization (Fig. 5). These observations suggest that the MOP catabolic system of strain NA513 also accepts MOA, but that this system has more affinity for MOP than for MOA.

To grow on the mannityl opine analogs, a bacterium must first cleave the original substrate and then catabolize at least one of the products of this initial reaction. The second requirement is at best partially fulfilled in the case of utilization of the deoxyalditYl analogs by strain MBA209. Detection of intermediates and end products of catabolism shows that, as for *Agrobacterium* spp., products of the initial cleavage of the analogs by this bacterium are the sugar and amino acid constituents of the molecule. In the case of the MOP analogs, glutamine is then converted to glutamate, which under the conditions of the assay cannot be degraded further. Thus, strain MBA209 relies solely on the sugar moiety for growth on the analog, and in the case of XYL-GLN at least the capacity for sugar catabolism, rather than specificity of the MOP catabolic system, was identified as responsible for the lack of complete utilization.

Mannose, glutamine, and glutamate can all be utilized by strain NA513, either when present alone (Table 3) or as components of MOP or MOA. This bacterium shows specificity for the deoxymannityl portion of MOP and MOA, utilizing only 1 of the 14 deoxyalditYl analogs tested. The level of specificity for the amino acid portion of the mannityl opines was comparable for strains B6S3 and NA513 (Table 5). In a separate study, a bacterium containing pRi8196 also utilized several amino acid analogs (11).

The postulated substrate specificity of the strain NA513 catabolic system (see above) was characterized in three ways. First, utilization of [^{14}C]MOP by cells of NA513 depends on pregrowth on a mannityl opine substrate (Fig. 3B). While this requirement is no longer expressed by the variant NA513B (Fig. 4), the catabolic potential of this variant remains virtually unaltered (Table 5). In contrast, spontaneous catabolic *Agrobacterium* mutants exhibited a markedly decreased specificity compared with the wild type (11). Second, incubating NA513 cells in a combination of MOP and analog yields results identical to those obtained with NA513B (Table 5). Third, mannose, glutamine, and several deoxyalditYl or amino acid analogs of MOP and MOA have no effect on MOP utilization by NA513 cells pregrown on MOP, with competitive inhibition being observed only with the two amino acid analogs nor-MOP and MAN-AIB. These results suggest the existence of specificity of the MOP catabolic system at the substrate affinity level.

The specificity exhibited by strain NA513 for the deoxyalditYl portion of MOP (Table 5) is accompanied by a weak potential for aldose utilization (Table 2). The possibility of a causal relationship between these two properties cannot formally be excluded.

Mannityl opines are specific to crown galls and hairy roots. The ability of the nonagrobacteria studied here to colonize these niches is not known. Thus, no hypothesis can be made as to the nature of the selection pressure which leads to acquisition of the mannityl opine utilization phenotype by *Arthrobacter* sp. and *P. putida*. Nevertheless, we postulate that the observed characters of the mannityl opine utilization systems in strains B6S3 and NA513 relate to the natural environment of these bacteria. The highly virulent strain B6S3 is likely frequently to encounter mannityl opines in nature. In contrast with this, MOP and MOA utilization by strain NA513, which does not induce crown galls or hairy roots, is mediated by a system specifically aimed at occasional substrates.

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