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Cultivation of Bacteria Producing Polyamino Acids with Liquid Manure as Carbon and Nitrogen Source

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Poly(γ-D-glutamic acid) (PGA)-producing strains of *Bacillus* species were investigated to determine their ability to contribute to reducing the amount of ammonium nitrogen in liquid manures and their ability to convert some of the ammonium into this polyamino acid as a transient depot for nitrogen. Organisms that do these things should help solve the serious environmental problems which are caused by the use of large amounts of liquid manure resulting from intensified agriculture; these problems are mainly due to the high content of ammonium nitrogen. *Bacillus licheniformis* ATCC 9945 and *Bacillus subtilis* were able to grow in liquid manure and to produce PGA in the presence of sodium gluconate. On artificial liquid manure these two strains were able to produce 0.85 and 0.79 g of PGA per liter, respectively. Under conditions that are found in intensified farming situations the ammonia content was reduced within 48 h from 1.3 to 0.75 g/liter. One mutant of *B. subtilis* 1551 impaired in the catabolism of PGA was obtained after nitrosoguanidine mutagenesis. This mutant produced PGA at a final concentration of 4.8 g/liter, whereas the wild type produced only 3.7 g/liter.

As a result of intensified agriculture, huge amounts of liquid manure are produced. On a typical pig-fattening farm (700 animals) in northwest Germany 2.8 m^3 is produced daily (European Communities website, http://europa.eu.int). Liquid manure is a highly heterogeneous liquid mixture of fodder, litter, and animal cells, and the composition is extremely dependent on the age of the pigs, the type of fodder, the age of the liquid manure, and the storage conditions (8). Average values for the major parameters of this manure have been shown to be follows (20): dry weight, 60 to 80 g/liter; chemical oxygen demand, 50 to 80, g/liter; biological oxygen demand, 15 to 32 g/liter; nitrogen concentration, 5.0 to 6.0 g/liter; ammonium nitrogen concentration, 2.5 to 4.5 g/liter; potassium concentration, 2.1 to 2.5 g/liter; calcium concentration, 1.8 to 2.1 g/liter; phosphorus concentration, 0.7 to 0.9 g/liter; and magnesium concentration, 0.5 to 0.6 g/liter.

The traditional and most widely used way to dispose of liquid manure is by spreading it onto agricultural fields. Up to 80% of the ammonia is released into the atmosphere, and up to 56% (wt/vol) of the remaining ammonia can be utilized by plants (12, 19). In addition, depending on the type of soil and the microflora, the ammonium ions are partially oxidized to nitrate. Up to 40% (wt/wt) of the nitrate is released into the groundwater or washed into the surface water, which leads to unacceptably high concentrations of nitrate and eutrophication (1, 6). This causes serious health and environmental problems and has forced legislators to restrict the amount of nitrogen in liquid manure that is spread onto fields; in Germany the amount is restricted to 210 kg per ha per year (3). Therefore, new methods for disposal of liquid manure from intensified animal-fattening farms are needed.

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Several gram-positive bacteria, such as *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus anthracis*, *Sporosarcina halophila*, and *Planococcus halophila*, are able to synthesize the polyamino acid (PAA) poly(γ-D-glutamic acid) (PGA) as a capsular substance or as a water-soluble slime (4, 22). In the presence of excess amounts of a suitable carbon source and ammonia, *B. licheniformis* excretes up to 20 g of PGA per liter into the medium (2). As PGA is remarkably resistant to proteolytic attack, degradation of this polymer by a nonadapted microflora proceeds very slowly (13).

In this study, we investigated conversion of the ammonia in liquid manure into PAAs by known PAA-producing bacteria. The PAAs should function as a transient depot for ammonia. Slow microbial degradation of the PAAs should then result in only low concentrations of free ammonia as a nutrient for plants. In addition, PAAs have a fertilizing function. Due to their polyanionic character, divalent cationic ions (i.e., Ca^{2+} and Mg^{2+}) bind to the polymer, and by this process they are obviously concentrated and more efficiently transferred to the rhizosphere of plants (7).

MATERIALS AND METHODS

Bacterial strains. All bacteria investigated in this study are listed in Table 1.

Growth of bacteria. *Bacillus* strains were cultivated at different temperatures in 0.8% (wt/vol) nutrient broth, in mineral salts medium (15), in liquid manure, in artificial liquid manure, or in particle-free liquid manure supplemented with carbon sources by using different rates of aeration as indicated below. Artificial liquid manure contained (per liter) 16.52 g of $(NH_4)_2SO_4$, 0.23 g of $Na_2HPO_4 \cdot 2H_2O$, 0.71 g of $MgSO_4 \cdot 7H_2O$, and 1.34 g of KCl (19). The pH was adjusted to 7.5 with 1.0 N H_2SO_4. Liquid manure was obtained from the Landwirtschafliche Untersuchungs- und Forschungsanstalt (Oldenburg, Germany) and was stored at 4°C. Prior to use the liquid manure was filtered, centrifuged for 10 min at 3,000 × *g* to remove solid particles, and autoclaved. The chemical composition of the liquid manure was as follows: 0.46% (wt/wt) nitrogen, 0.32% (wt/wt) ammonium N, 0.27% (wt/wt) P_2O_5, 0.29% (wt/wt) K_2O, and 3.9% (wt/wt) dry matter. For PGA production medium E was also used (11). To obtain solid media, 1.5% (wt/vol) agar agar was added. Growth was monitored photometrically at 600 nm or with a Klett-Summerson photometer by using a green no. 54 filter (520 to 580 nm).

TABLE 1. Bacterial strains used in this study

Bacterial strain	Other designation[a]	Reference
Bacillus licheniformis ATCC 9945	SK7085	22
Bacillus subtilis 1551	SK5668	
Bacillus subtilis b′5	SK5665	
Bacillus subtilis DSM10		5
Bacillus subtilis natto IFO3335		9
Bacillus subtilis GM273	SK5295	
Bacillus subtilis GM329	SK5296	
Bacillus subtilis S35	SK5664	
Bacillus subtilis MP5	SK7889	
Streptomyces albulus 346		17

[a] Our strain collection designation.

Nitrosoguanidine mutagenesis. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis of *B. subtilis* 1551 was performed as described previously by Reh and Schlegel (14).

Electrophoretic methods. Proteins were separated under denaturing conditions in 11.5% (wt/vol) polyacrylamide gels by the method of Laemmli (10) and were stained with Serva Blue R.

Quantitative determination of ammonia. The ammonia content was determined with a type 15 230 3000 gas-sensitive electrode (Mettler Toledo GmbH, Steinbach/Ts, Germany) according to the instructions provided by the manufacturer.

Qualitative and quantitative determination of PAAs. The amount of isolated PAAs was analyzed after precolumn derivatization of PAA hydrolysates with *ortho*-phthaldialdehyde reagent on a reversed-phase high-performance liquid chromatography column as recommended by the manufacturer (Merck, Darmstadt, Germany) (21). Hydrolysis was carried out by adding 1 ml of 6 N HCl to 1 mg of lyophilized PAAs and incubating the mixture for 6 h at 105°C.

RESULTS

Search for suitable strains able to produce PAAs in liquid manure. In order to identify organisms suitable for conversion of the ammonia that is present in liquid manure into PAAs, strains of bacterial species which had been found previously to excrete PGA, including *B. licheniformis* ATCC 9945 and *B. subtilis natto* IFO3335, as well as polylysine-producing *Streptomyces albulus* strain 346, were tested for PAA production under the appropriate optimal conditions (Table 2). Whereas *B. licheniformis* ATCC 9945 and *B. subtilis* (*natto*) IFO3335 showed the expected PGA production, *S. albulus* 346 did not excrete any detectable polylysine. In addition, six other *Bacillus* strains from the culture collection of our laboratory were analyzed for PAA production. These studies identified two of these six strains as PGA producers.

The PGA-producing strains *B. licheniformis* ATCC 9945, *B. subtilis* (*natto*) IFO3335, and *B. subtilis* 1551 and b′5 were chosen to investigate the influence of liquid manure on polymer production. To do this, the production medium was replaced stepwise by increasing the fraction (33, 50, 66, or 100%, [vol/vol]) of liquid manure. Although all strains produced reasonable amounts of PGA in the absence of liquid manure, the amounts of PGA were significantly reduced in the presence of increasing concentrations of manure, as shown in Fig. 1.

TABLE 2. Production of PAA by bacteria on solid medium E

Bacterial strain	PAA production[a]
Bacillus licheniformis ATCC 9945	+++
Bacillus subtilis 1551	+++
Bacillus subtilis b′5	+
Bacillus subtilis DSM10	(+)
Bacillus subtilis (*natto*) IFO3335	−
Bacillus subtilis GM273	−
Bacillus subtilis GM329	−
Bacillus subtilis S35	++
Bacillus subtilis MP5	++
Streptomyces albulus 346	−

[a] Organisms were cultivated for 24 h at 35°C. PAA production: +++ > ++ > + > (+). −, no PAA production.

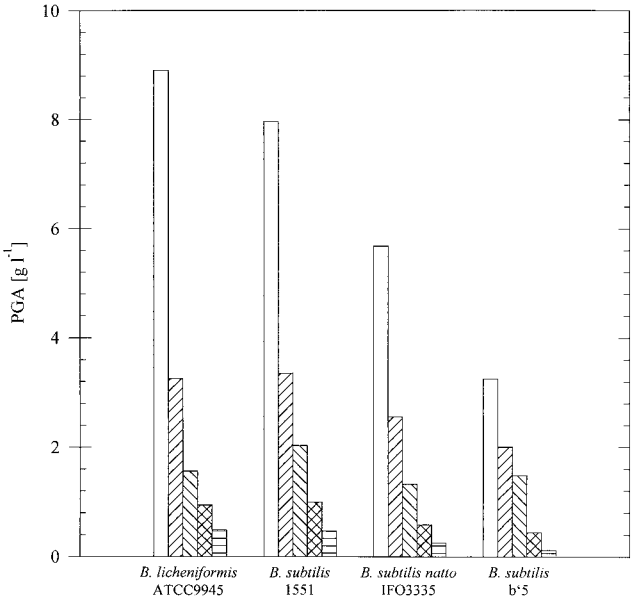

FIG. 1. Cultivation of several *Bacillus* strains with different concentrations of liquid manure. The strains were cultivated at 35°C for 96 h in 500-ml conical flasks equipped with three baffles containing a total volume of 100 ml of medium. Medium E was replaced stepwise by increasing the fraction of liquid manure, as follows: 0% (vol/vol) (□), 33% (vol/vol) (▨), 50% (vol/vol) (▧), 66% (vol/vol) (▩), and 100% (vol/vol) (▤).

Effects of different carbon sources on PGA production. In liquid manure, the nitrogen-to-carbon ratio is approximately 11:1 (16). In medium E, which provides the optimal amounts of carbon and nitrogen for PGA production by *B. licheniformis* ATCC 9945 (22), the nitrogen-to-carbon ratio is 1:13.8. Therefore, liquid manure was supplemented with different relatively inexpensive carbon sources to improve the conditions for PGA production.

As Table 3 shows, good growth and PGA production were obtained with both *B. licheniformis* ATCC 9945 and *B. subtilis* 1551 on liquid manure in the presence of sodium gluconate. Glucose and sucrose also had positive effects on growth and PGA production. Beet molasses stimulated growth only slightly and did not or almost did not result in production of PGA. Addition of whey also had a negligible effect. Since these carbon sources had no effect or only a negligible effect on growth of and PGA production by the other two strains of *B. subtilis* and also had no effect on growth of and polylysine production by *S. albulus*, *B. licheniformis* ATCC 9945 and *B. subtilis* 1551 were chosen for further detailed studies of the growth kinetics.

Determination of growth kinetics by using artificial and particle-free liquid manure. Differences in the composition of liquid manure made it impossible to obtain reproducible quan-

TABLE 3. Growth of and PAA production by different bacteria on liquid manure plates with several carbon sources

Strain	Glucose[a]		Sodium gluconate		Sucrose		Beet molasses		Whey		No carbon source	
	Growth	PAA production	Growth	PAA production	Growth	PAA production	Growth	PAA production	Growth	PAA production	Growth	PAA production
B. licheniformis ATCC 9945	++	+	+++	+++	++	+	++	(+)	−	−	(+)	−
B. subtilis 1551	++	+	+++	+++	++	+	+	−	(+)	−	(+)	−
B. subtilis b′5	+	−	+	−	−	−	−	−	−	−	−	−
S. albulus 346	−	−	−	−	−	−	−	−	−	−	−	−
B. subtilis (*natto*) IFO3335	+	(+)	+	(+)	−	−	−	−	−	−	−	−

[a] The strains were cultivated for 72 h at 35°C on liquid manure plates containing a carbon source at a concentration of 0.5% (wt/vol). Growth and PAA production: +++ > ++ > + > (+). −, no growth or no PAA production.

titative data for growth and conversion of ammonia into PGA. Furthermore, liquid manure contains up to 4.7% (wt/vol) solid material (e.g., hairs, straw, etc.), which prevented continuous measurement of growth and PGA content. Therefore, at the beginning of the experiments, artificial liquid manure was used as the medium for comparative studies of the growth kinetics of *B. licheniformis* ATCC 9945 and *B. subtilis* 1551. The composition of artificial liquid manure corresponded to the average values for the main soluble mineral constituents of liquid manure (19).

On artificial liquid manure containing 0.5% (wt/vol) sodium gluconate, both *B. licheniformis* ATCC 9945 (Fig. 2A) and *B. subtilis* 1551 (Fig. 2B) exhibited relatively fast growth, with doubling times of 3.2 and 2.4 h, respectively. PGA production started after the cells entered the stationary growth phase, and the concentrations reached maximum values of 0.85 and 0.79 g/liter, respectively, after 96 h of cultivation. Slower growth (doubling times, 13.0 and 10.8 h, respectively) and less PGA (0.58 and 0.39 g/liter, respectively) were obtained with these strains in the presence of 0.5% (wt/vol) glucose. No PGA production was detected during cultivation of either strain on sucrose, beet molasses, or whey. Whereas *B. licheniformis* ATCC 9945 exhibited weak growth on sucrose (doubling time, 6.6 h) and even less growth on molasses, *B. subtilis* 1551 was not able to use either substrate for growth. With both species, no growth occurred in the presence of whey.

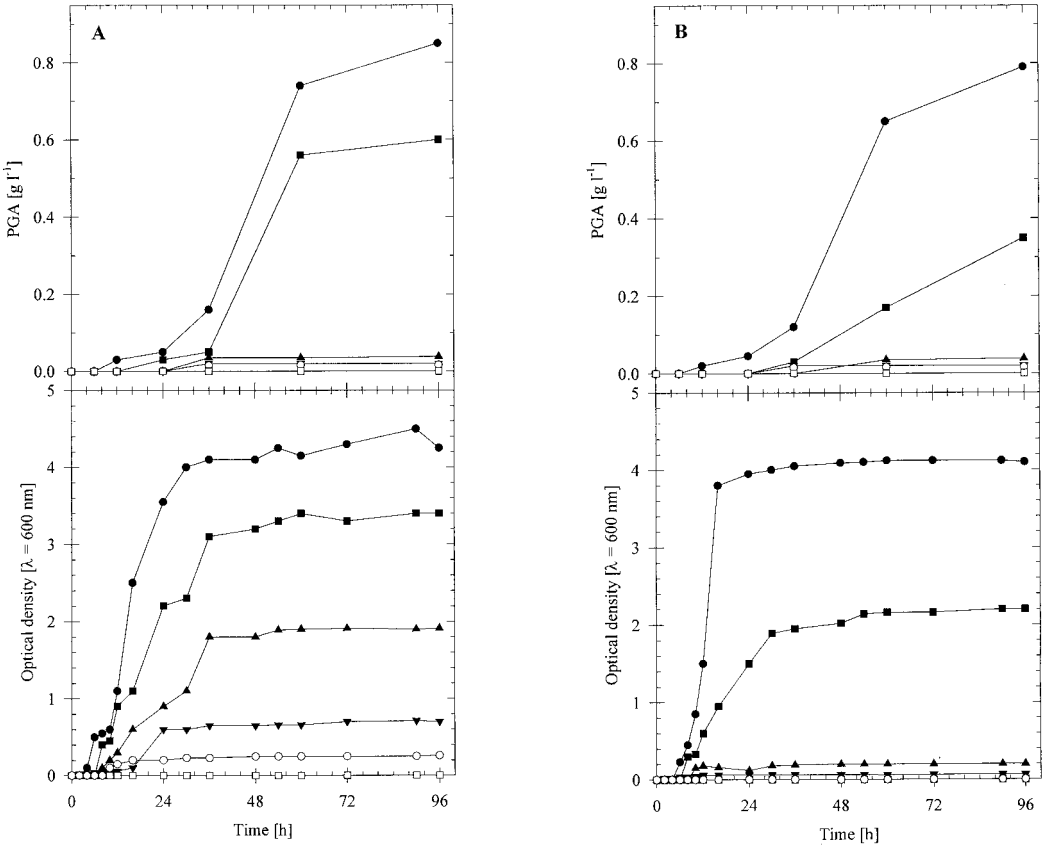

FIG. 2. PGA production and cultivation of *B. licheniformis* ATCC 9945 (A) and *B. subtilis* 1551 (B) in artificial liquid manure. The strains were cultivated at 35°C for 96 h in 500-ml conical flasks equipped with three baffles containing 100 ml of artificial liquid manure. The concentration of each carbon source was 0.5% (wt/vol). Symbols: ●, sodium gluconate; ■, glucose; ▲, sucrose; ▼, beet molasses; ○, whey; □, no carbon source.

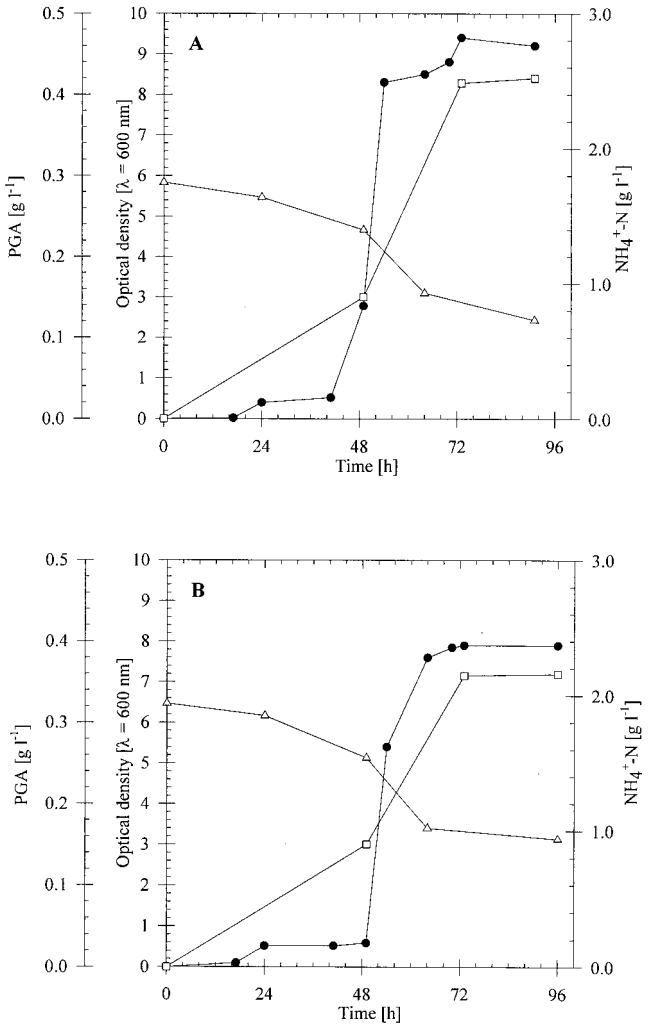

FIG. 3. Cultivation of, ammonium reduction by, and PGA production by *B. licheniformis* ATCC 9945 (A) and *B. subtilis* 1551 (B) in particle-free liquid manure. The strains were cultivated at 35°C for 96 h in 500-ml conical flasks equipped with three baffles containing 100 ml of particle-free liquid manure. The concentration of sodium gluconate was 0.5% (wt/vol). Symbols: ●, optical density at 600 nm; △, ammonium concentration; □, PGA concentration.

In order to verify these results under conditions more likely to occur in the field, natural liquid manure was used, but it was pretreated by filtration and centrifugation to obtain particle-free liquid manure. During cultivation of *B. licheniformis* ATCC 9945 and *B. subtilis* 1551 in particle-free liquid manure, which was supplemented with 0.5% (wt/vol) sodium gluconate, PGA production and reduction of ammonium occurred at rates which were 50% of those obtained with artificial liquid manure (Fig. 3). As revealed by comparison with a sterile control that was incubated under identical conditions, approximately 40% of the reduction was due to evaporation of NH_3. Therefore, based on a PGA yield of approximately 0.4 g/liter, only 5% of the ammonia in each case was converted into PGA.

Conversion of ammonia into PGA under conditions that occur in intensified farming situations. In order to test the ability of the PGA-producing bacteria to convert ammonia into the PGA polymer under conditions similar to those that occur in intensified agriculture situations, growth experiments were performed in a medium composed of 54% (vol/vol) tap water, 33% (vol/vol) medium E, and 13% (vol/vol) particle-free liquid manure. This composition resembled those that were used in a pilot plant at the Landwirtschaftliche Untersuchungs- und Forschungsanstalt. In this pilot plant, experiments to reduce the ammonia content of liquid manure by nitrification were done, and to 0.13 volume of liquid manure 0.33 volume of activated sludge and 0.54 volume of water were added. Once a week 0.13 volume of the culture fluid was replaced by fresh liquid manure.

As shown in Fig. 4, *B. licheniformis* ATCC 9945 and *B. subtilis* 1551 exhibited similar growth under the conditions mentioned above. After inoculation with *B. licheniformis* ATCC 9945, the ammonia content was reduced from 1.3 to 0.75 g/liter within 48 h. In two steps after 48 and 96 h of cultivation, 0.13 volume of the culture volume was replaced by fresh liquid manure. During the following 2 days of incubation, the ammonia concentration was reduced to 0.74 g/liter. Net production of PGA occurred only during the first 96 h, and the maximum value was 2.2 g/liter. During the following 2 days, the concentration of PGA decreased to 1.6 g/liter.

The results obtained with *B. subtilis* 1551 differed from those obtained with *B. licheniformis*; both reduction of ammonia and production of PGA were lower (Fig. 4).

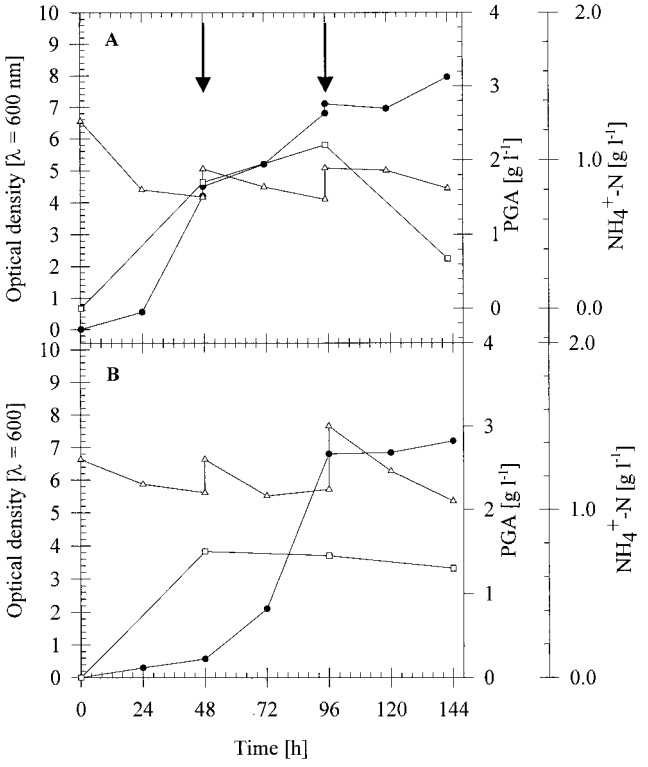

FIG. 4. Cultivation of *B. licheniformis* ATCC 9945 (A) and *B. subtilis* 1551 (B) under fed batch conditions. The strains were cultivated at 35°C for 144 h in 500-ml conical flasks equipped with three baffles containing 100 ml of medium composed of 54% (vol/vol) tap water, 33% (vol/vol) medium E, and 13% (vol/vol) particle-free liquid manure. At the times indicated by arrows, 0.13 volume of particle-free liquid manure was added. Symbols: ●, optical density at 600 nm; △, ammonium concentration; □, PGA concentration.

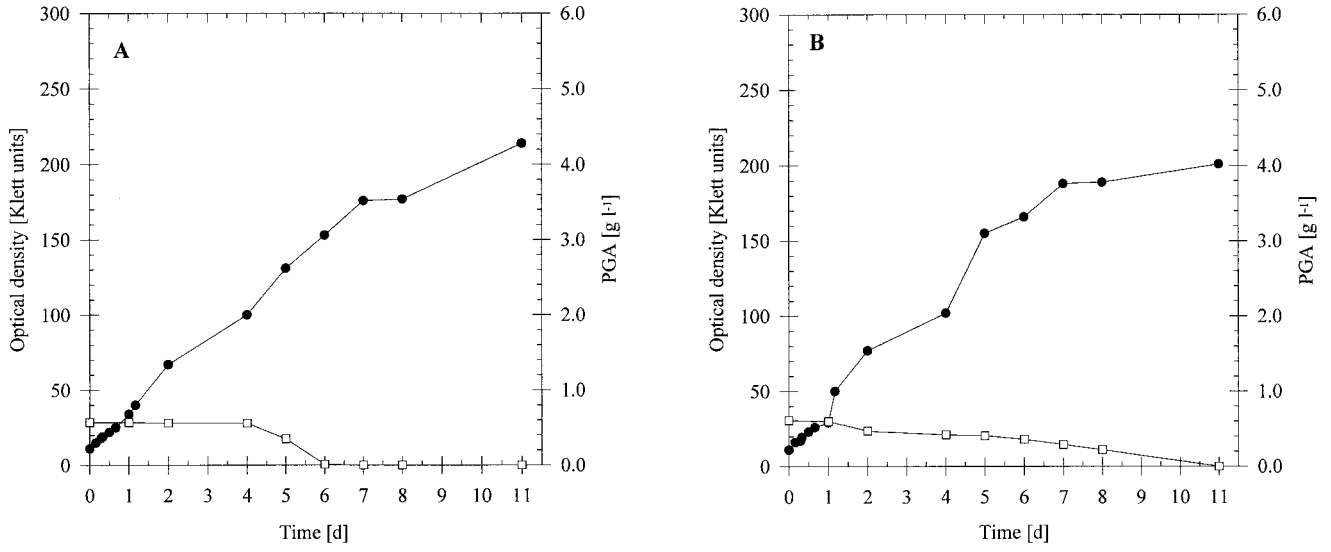

FIG. 5. Consumption of PGA by *B. subtilis* 1551 (A) and the PGA-degrading mutant *B. subtilis* MP5 (B) in mineral salts medium. The strains were cultivated at 35°C for 11 days in 500-ml conical flasks equipped with three baffles containing 100 ml of mineral salts medium and 0.5% (wt/vol) PGA as the sole carbon source. Symbols: ●, optical density as measured with a Klett-Summerson colorimeter; □, PGA concentration.

Isolation and characterization of a *B. subtilis* mutant affected in degradation of PGA. PGA-producing bacteria are generally also able to utilize the excreted PGA polymer as a nutrient (13). Therefore, mutants of PGA-producing strains affected in degradation of PGA should give higher yields of the polymer during batch cultivation in liquid manure. After mutagenization of *B. licheniformis* ATCC 9945 and *B. subtilis* 1551 with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, the cells were incubated in 50 ml of nutrient broth in the presence of penicillin (75 mg/ml) to enrich those mutants which were defective in degrading PGA. To identify and isolate the desired mutants, the cells were plated on medium E, on which slimy colonies indicated the wild type by the ability to excrete PGA. In parallel, the cells were also plated onto mineral medium with 0.5% (wt/vol) PGA as the sole carbon source, on which no or reduced growth indicated defective PGA degradation.

Although no mutant of *B. licheniformis* ATCC 9945 with the desired phenotype was obtained, one stable mutant of *B. subtilis* 1551 out of 1,500 candidates investigated showed reduced growth on PGA. Cultivation in liquid medium containing PGA as the sole carbon source revealed that this mutant, which was referred to as MP5, was able to grow on the polymer, but the rate of degradation was significantly lower than that of the wild type (Fig. 5). Thus, mutant MP5 exhibited the phenotype PGA leaky. This reduced capacity resulted in faster polymer production and a higher yield during cultivation in medium E, in

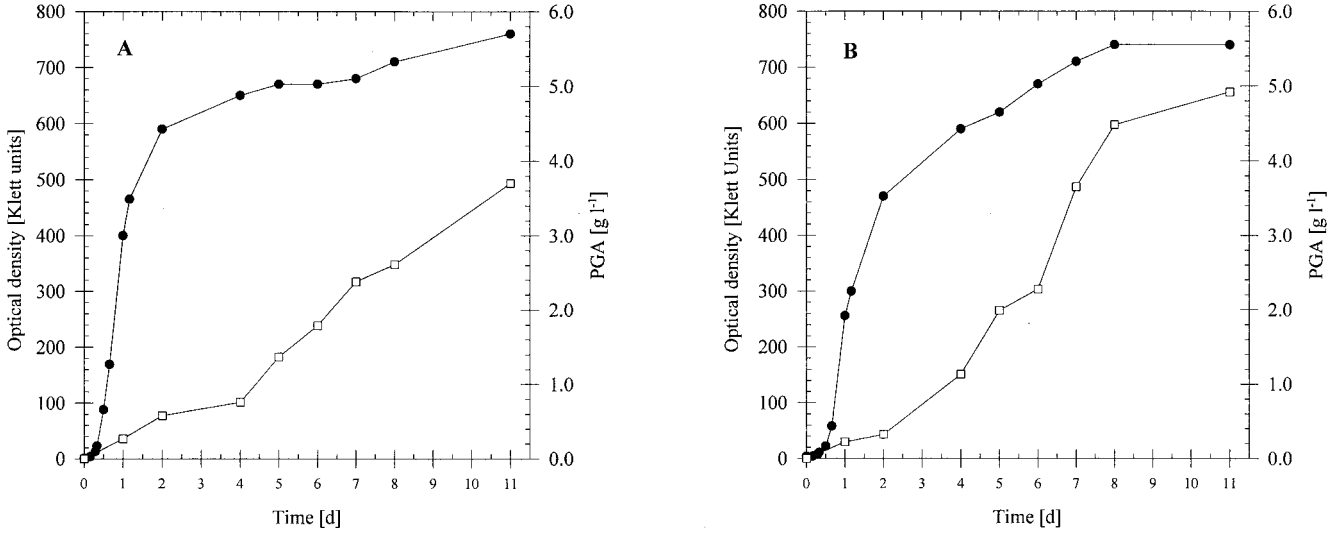

FIG. 6. Production of PGA by *B. subtilis* 1551 (A) and the PGA-degrading mutant *B. subtilis* MP5 (B) in medium E. The strains were cultivated done at 35°C for 11 days in 500-ml conical flasks equipped with three baffles containing 100 ml of medium E. Symbols: ●, optical density as measured with a Klett-Summerson colorimeter; □, PGA concentration.

which the mutant produced PGA at final concentrations up to 4.8 g/liter, whereas the final concentration of PGA obtained with the wild type was only 3.7 g/liter (Fig. 6).

Polyacrylamide gel electrophoresis of denatured crude extracts of mutant MP5 and the wild type revealed a clear difference. Whereas the protein pattern for cells of the wild type grown on PGA had a weak but distinct band representing a protein with an apparent M_r of 35,000 ± 1,000, which was absent in wild-type cells after growth in PGA production medium, this protein was absent in the electropherograms of mutant MP5 cells in the early growth phase and in the late stationary growth phase.

DISCUSSION

The purpose of this study was to describe a process for conversion of the ammonia of liquid manure into PAAs. In this study, it was shown that PGA-producing species of the genus *Bacillus* are able to convert a significant fraction of the ammonium nitrogen present in liquid manure transiently into this PAA. By this process, the concentration of free ammonium in the manure could be reduced, and the PGA produced acted as a transient depot for ammonium. However, the conversion occurred only to a low extent. In the case of *B. licheniformis* ATCC 9945 and *B. subtilis* 1551 this was mainly due to the carbon-to-nitrogen ratio in liquid manure, which is the inverse of that in medium E, which is known to favor PGA production. Consequently, an increase in PGA production with a corresponding reduction in the amount of ammonia was achieved by adding a suitable carbon source or medium E to liquid manure. Another strategy involving a better nitrogen sink is application of organisms producing the proteinlike polymer cyanophycin, which consists of equimolar amounts of arginine and aspartic acid (nitrogen to carbon ratio, 1:2) (18). Cyanophycin is unique to cyanobacteria and has been reported to occur in many species of cyanobacteria. At present, the use of genetically engineered microorganisms would be necessary to produce such a nitrogen storage compound in manure. Further systematic optimization of the culture conditions would surely lead to improvement of the conversion rate. Whether such a strategy can be biotechnologically applied will depend on further optimization of the process and also on isolation of PGA-producing bacteria that are better adapted to the conditions prevailing at agricultural sites, including high concentrations of manure and relatively low temperatures.

However, any technical process for refinement of liquid manure must comply with the requirements of agriculture and consequently should be as simple and cheap as possible. Both treatment of liquid manure to improve the culture conditions and control of the process may be too costly and too sophisticated to be accepted by farmers.

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