Salt-Inducible Bionylon Polymer from *Bacillus megaterium*<sup>▼</sup>

Kazuki Shimizu, Hisaaki Nakamura, and Makoto Ashiuchi*  
Department of Bioresources Science, Kochi University, Nankoku, Kochi 783-8502, Japan  
Received 16 November 2006/Accepted 27 January 2007

Poly-γ-glutamate (PGA) is a chiral polyamide material that possesses a nylon-like backbone, a bionylon polymer. We examined the PGA productivity of *Bacillus megaterium* and found NaCl-responsive PGA production in the bacterium. In the system of *B. megaterium*, salt would be significant in controlling the yield, molecular size, and stereochemistry of bionylon.

Poly-γ-glutamate (PGA) is a biopolymer with a nylon-like backbone, and some experiments regarding the esterification of its α-carboxyl side chains indicate that PGA substantially exhibits nylon-like properties (11). Unlike synthetic nylons, PGA with multiple chirotopic carbons possesses fair biodegradability and other useful functionalities (1). Therefore, we have tentatively designated PGA a bionylon. PGA-based biopolymers are mainly produced by bacilli, e.g., *Bacillus subtilis*, *B. anthracis*, and *B. megaterium* (1, 2); *B. subtilis* produces the D/L-copolymer type (D-glutamate content, 60% ± 15%; L-isomer content, 40% ± 15%), whereas *B. anthracis* produces the D-rich polymer type (D-glutamate content, nearly 100%). In contrast, the polymer productivity of *B. megaterium* remains obscure.

In this study, *B. megaterium* WH320 (MoBiTeC Co., Göttingen, Germany) was used; like *B. subtilis*, *B. megaterium* is not halophilic. *B. megaterium* was first cultured in 200 ml of LB medium (14) at 37°C for 24 h, and growing cells were collected by centrifugation at 8,000 × g for 15 min. Harvested cells were resuspended in 2 ml of saline. A 0.5-ml aliquot of cell suspension was inoculated into 50 ml of LG medium, which is often used for PGA production by *B. subtilis* (2), and cultured at 37°C for 5 days. Isolation and determination of PGA were carried out by the methods of Park et al. (13). Although *B. megaterium* never accumulated the exopolymer in salt-poor liquid media such as LG medium (Fig. 1A), increasing the salt concentration, e.g., more than 2% NaCl (wt/vol), resulted in a dramatic increase in the polymer yield. The results indicated that the maximum volumetric yield of PGA from liquid cultures of *B. megaterium* is 8.6 mg/ml, which is almost as high as those of typical *B. subtilis* PGA over-producers (1, 5). Then, sodium dodecyl sulfate-polyacylamide gel electrophoresis analysis of PGA (4, 13) was used for visualization of the polymer that accumulated in the salt-rich liquid medium (Fig. 1B). It is noteworthy that PGA with a greater molecular size was obtained from a medium containing a higher salt concentration. On the basis of determinations of the molecular size of PGA (13, 15), the average molecular masses of the polymer in 5 and 10% NaCl-containing media were estimated to be 1,000 and >2,000 kDa, respectively. To our knowledge, this is the first finding of a bacterium with NaCl-responsive PGA production, which may lead to a simple strategy for controlling the synthesis of bionylon.

Furthermore, the stereochemistry, or D/L ratio, of *B. megaterium* PGA was examined. When a liquid medium containing a high concentration of salt was used, an L-rich polymer in which the L-glutamate content reached a maximum of 95% was obtained consistently (Table 1). In Table 1, the PGA production data are represented as the polymer productivity of *B. megaterium* cells so that the effects of culture conditions can be

---

<sup>▼</sup> Corresponding author. Mailing address: Department of Bioresources Science, Kochi University, Nankoku, Kochi 783-8502, Japan. Phone: 81-88-864-5215. Fax: 81-88-864-5200. E-mail: ashiuchi@cc.kochi-u.ac.jp.

<sup>∗</sup> Published ahead of print on 9 February 2007.

**FIG. 1.** Salt-responsive PGA production by *B. megaterium*. (A) PGA accumulation in modified LG medium containing the indicated concentrations of NaCl (0 to 10%). Standard LG medium (2) contains 0.05% NaCl. Isolated PGA was first hydrolyzed with distilled HCl (6 M) at 105°C for 8 h in vacuo (4, 5). The total amount of glutamate monomers thus generated, which is applicable to the calculation of the polymer yield (3), was determined by means of high-performance liquid chromatography with a chiral separation column (3–5); typically, D-glutamate is more rapidly eluted than L-glutamate. The standard curves for D- and L-glutamate (showing the relationships of the amounts and the apparent peak areas of the high-performance liquid chromatography profiles) are *y* = 2.97x (fmol) and *y* = 2.91x (fmol), where *x* represents each peak area. (B) Sodium dodecyl sulfate-polyacylamide gel electrophoresis and visualization of PGA. PGA (an acidic polymer) was specifically visualized by staining the gel with methylene blue (a basic dye) (1, 3–5); in contrast, typical protein polypeptides are not stained by this dye. Each band corresponds to the amount of PGA that accumulated in 0.3 ml of culture medium in the absence (lane a) or presence of NaCl at 0.05% (lane b), 0.2% (lane c), 0.5% (lane d), 1% (lane e), 2% (lane f), 5% (lane g), or 10% (lane h). Lane M, molecular size markers. These observations were verified by double checking.
assessed. Indeed, a bionylon polymer with a similar proportion was produced on solid medium containing a high concentration of salt as well; however, the productivity of cells grown on the solid medium was changeable and essentially lower than that of cells grown in the liquid medium. We thus succeeded in the effective production of an L-rich polymer-type bionylon by using a liquid Bacillus culture system. On the other hand, the production of the DL-copolymer-type bionylon (D-glutamate content, 30% ± 5%; L-isomer content, 70% ± 5%) by *B. megaterium* on an agar plate of LG medium (containing a low concentration of salt [2]) has been considered (2, 3). Therefore, our observations suggest that salt as a determinant of the structural features (e.g., molecular size and stereochemistry) of bionylon in *B. megaterium*. 

*B. megaterium* requires a large amount of L-glutamate for polymer production (data not shown), as does a group of *B. subtilis* PGA overproducers (1, 5), whereas it possesses only a little r-glutamate-supplying activity (e.g., glutamate racemase, <1 mU/mg of cytosolic protein; r-amino acid aminotransferase, 0 mU/mg; reference 5), viz., a point of resemblance to t-rich PGA-producing hyperhalophilic archaea (1, 6) with no d-glutamate productivit.

Bionylon, especially the polymer with a stereoregular structure, such as t-rich PGA from archaea (6), is versatile. Archaeal PGA (t form) may be far superior to *B. subtilis* PGA (d form) even in biochemical applications (e.g., enzyme stabilization [M. Ashiuchi and D. Yamasaki, September 2005, Japanese patent pending]). The very low PGA productivity of archaea under liquid culture conditions (2, 7), however, remains to be improved in order to ensure the feasibility of bionylon. Here we conclude that *B. megaterium* has the potential to address these limitations and hence exhibits industrial applicability as a supplier of such new biodegradable materials. Interestingly, although it had been revealed that the production of exopolymere-substances (mainly polysaccharides) was strongly suppressed by salt in a halotolerant strain of the bacterium *Rhizobium meliloti* (10), *B. megaterium* produced extracellular t-rich PGA in response to the salt concentration in the medium (Fig. 1A), as did hyperhalophilic archaea (6, 7). This finding suggests that a bionylon polymer with the desired stereochemistry may possess extremely-like functionality (9). Figure 1B corresponds to the first example of the establishment of molecular-size-controlled PGA production, while a recent report presented the special functions of super-high-molecular-weight PGA (15). In addition, some studies have demonstrated that living organisms other than bacilli (4, 8, 12, 15), even a higher plant (16), can be briefly endowed with PGA productivity by genetic manipulation. We are now investigating the molecular machinery that directs the synthesis of the salt-inducible bionylon polymer in *B. megaterium*, and its identifi-cation and application will probably provide insights into new biotechnology using chiral polypeptide materials.

### REFERENCES