Making the selective agent for the *Bar* plasmids, phosphinothricin (glufosinate) affordable for routine use

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Phosphinothricin (glufosinate) is an antimetabolite that has been used for the selection of fungal transformants carrying the *Bar* plasmid and its various derivatives. The cost of the pure material has limited its usefulness as a selective agent We describe a purification of glufosinate from an inexpensive commercial source.

Plasmids carrying dominant antimetabolite resistance markers are widely useful in molecular genetics, but the choice of suitable antimetabolites has been small. For example, benomyl is an effective antimetabolite, but transformants to benomyl resistance are often sterile (Staben et al 1989 Fungal Genet. Newsl. **36**: 79-81), whereas hygromycin is expensive, as well as toxic. The *Bar* family of plasmids, which confer resistance to the glutamine analog glufosinate, are potentially very useful as cloning vectors (Pall 1993 Fungal Genet. Newsl. **40**:58; Pall and Brunelli *ibid*. 59-63; 1994, *ibid*.**41**: 63-65; Sweigard et al. 1995, *ibid* **44**: 52-53), but the high cost of glufosinate (as the ammonium salt) has been an important consideration in the design of experiments: the price ranges from $360 per gram from one chemical company to over $2,000 per gram from a major supplier of laboratory reagents. Since concentrations in the range of 200 µg/ml must be used to obtain clean selection, the cost per Petri dish is $1.80 to $10.00 -- prohibitive for many experiments.

The herbicide Finale, sold by AgrEvo Environmental Health, 95 Chesmut Ridge Road, Montvale NJ 07645, is available at nurseries for about $25 per 1090 ml, and contains 63 g of ammonium glufosinate. The street value of this quantity of the pure compound, is $22,500 to $126,000, a handsome markup by any standard. Unfortunately, Finale can not be used directly because it also contains detergents and other additives that are lethal to *Neurospora* and probably to other fungi. The following simple procedure gives a quantitative or near quantitative yield of ammonium glufosinate from Finale, allowing the researcher to capture the 900-5000 fold markup for other projects.

A glass column, internal diameter 2.5 cm x 50 cm, was filled to a depth of 44 cm. by slurrying in a water suspension of Dowex-50-H$^+$, 8% crosslinked (BioRad, 200-400 mesh). One column volume of Finale (215 ml) was added dropwise at the top, and when all had sunk into the resin, the column was eluted with 370 ml of 1.0 M acetic acid. The acetic acid was followed by 50 ml of water. These combined washings, which contained much of the detergent and some blue dye, were ninhydrin-negative, i.e., contained no glufosinate, and were discarded. Elution was then started with 4.0 M ammonium hydroxide. The dark yellow (H+) form of the resin was discharged to a brown color as the front of the ammonium form advanced. At the moment the brown front reached the bottom of the column, which required about 130 ml of ammonium hydroxide, the pH of the eluate, as measured by pH paper, increased within the interval of a few drops from about 5 to about 10, and the first glufosinate appeared in the eluate. The next 80 ml was collected in a batch and contained virtually all the glufosinate. It was evaporated in a large beaker in a good vacuum; a beaker of concentrated sulfuric acid accompanied it in the desiccator to take up the ammonia gas and water vapor. The syrup of ammonium glufosinate was taken up in water to a final volume of 62 ml (200 mg/ml). Charcoal (50 mg) was added to remove the trace of blue dye that remained in this fraction. The preparation was stirred for a few minutes and then filtered through a 0.45 µm filter to remove both charcoal and any remaining microorganisms. The preparation was stored frozen. The column was regenerated by using 250 ml of 2 M sodium acetate, followed by 4 M HCl until the eluate was qualitatively free of sodium ions, as judged by flaming a loopful. The column was then washed with water until the eluate was free of acid, as detected by pH paper, and was then ready for re-use.

The purified ammonium glufosinate was tested for effectiveness in medium made by adding 1 ml of the stock solution per liter (final concentration of 200 µg/ml) of autoclaved Vogel salts + agar medium from which ammonium nitrate was omitted and proline substituted at 5 mg/ml as the nitrogen source. The sugars, also added separately, were sorbose, glucose, and fructose (1%, 0.05%, and 0.05%, respectively). Conidia of wild type *N. crassa* were electroporated with covalently-closed plasmid pBARTME1 from the Fungal Genetics Stock Center by the method of Margolin, Freitag, and Selker (Fungal Genet. Newsl. 1997, **44**: 34-36), in a 1 mm gap cell with a potential drop of 1500 volts. Aliquots were plated in soft agar of otherwise the same composition as the bottom agar and incubated at 34°C. Transformation was efficient, in the range of 10^3 transformants per µg of plasmid. As observed previously by others, there were also some spontaneous resistant colonies on the no-DNA control plates -- in one trial, about 1.3% of the number on the corresponding experimental plates. There was no appreciable lawn of slow-growing, untransformed cells. Untransformed cells plated to lower concentrations of ammonium glufosinate (20 and 60 µg/ml) showed that these concentrations gave adequate inhibition for scoring and probably for selection of transformants, although there was a marginally noticeable background lawn and significantly more spontaneous resistant mutants.

We wondered whether we, and others, were not working at cross-purposes in preparing an ammonium-free basal medium and then adding ammonium glufosinate up to 200 µg/ml (about 1 mM). Therefore we prepared sodium glufosinate by running the ammonium salt through a Dowex-50-Na^+column. When inhibition by the sodium salt and ammonium salt were compared at identical molar concentrations, the sodium salt appeared to be very slightly more inhibitory, but not enough to justify even the small effort of preparing it from the ammonium salt.