ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1998, p. 228–230 Vol. 42, No. 2
0066-4804/98/$04.00+0

Chromogenic Detection of Aminoglycoside Phosphotransferases

ANA M. AMOROSO AND GABRIEL O. GUTKIND*

Laboratorio de Resistencia Microbiana, Cátedra de Microbiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

Received 14 May 1997/Returned for modification 14 August 1997/Accepted 18 November 1997

A coupled chromogenic reaction (based on an agar overlay combining NADH, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, ATP, and kanamycin sulfate with thiazolyl blue-phenazine methosulfate for detection of NADH consumption) was optimized for the detection of aminoglycoside phosphotransferases (APHs). When used after analytical isoelectrofocusing of bacterial extracts from APH-producing strains, this method revealed one band in each of two strains with a genetically confirmed APH (3′) I and two bands in another strain with both APH (3′) I and APH (3′) VI, whereas no bands were detected in susceptible control strains or in aminoglycoside-resistant microorganisms without APH genes.

The most common mechanism of acquired resistance to aminoglycosides in clinical isolates is that of aminoglycoside-modifying enzymes (3). These enzymes are capable of three general reactions: N acetylation, O nucleotidylation, and O phosphorylation (1). The modifying enzymes present in resistant bacteria may be studied by examining the resistance phenotype with substrate or nonsubstrate antibiotics, which merely provides an indication of enzyme types (5). However, as several different enzyme types (6) and isozymes may occur in an individual isolate, attempts to recognize each component may be difficult, since several resistance phenotypes may be superimposed.

Resistance phenotypes and hybridization with DNA probes specific for known enzymes provide a reliable assessment of resistance in an isolate (2). However, as the complete set of probes is available to only a few laboratories around the world, most laboratories are unable to test for them. Moreover, any putative new enzyme would be detected only after failing to hybridize to all known probes while carrying a new resistance pattern or a resistance pattern resembling that corresponding to a known gene.

In this study, an attempt was made to develop a coupled chromogenic system to detect aminoglycoside phosphotransferase (APH) activity in cell extracts after separation of the enzymes by analytical isoelectrofocusing, which is currently used for β-lactamase characterization.

MATERIALS AND METHODS

Resistant bacteria with phenotypically and genetically characterized enzymes were kindly provided by A. Rossi from the Institute C. G. Malbrán, Buenos Aires, Argentina. The strains and the genes encoding the modifying enzymes are as follows: *Proteus mirabilis* INM 8628, *aph* (3′)I + *ant* (2′); *P. mirabilis* INM 900, *aph* (3′)I + *aph* (3′)VI + *ant* (2′); *Escherichia coli* INM 7251, *aph* (3′)I + *aac* (6′); *E. coli* ATCC 11105, none; and *P. mirabilis* CCMA-29 1157, none (INM, Instituto Nacional de Microbiología Carlos Malbrán; ATCC, American Type Culture Collection; CCMA-29, Colección de Cultivos Microbianos, [Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires]).

Bacterial cells were grown to the late logarithmic phase in 500 ml of brain heart infusion (Merck Quimica Argentina) at 37°C in the presence of 50 μg of kanamycin (Armstrong) per ml, harvested by centrifugation (6,000 × *g*, 30 min, 4°C), resuspended in 10 mM Tris HCl–10 mM magnesium acetate–25 mM ammonium chloride, pH 7.8 (all chemicals were purchased from Merck Quimica

* Corresponding author. Mailing address: Laboratorio de Resistencia Microbiana, Cátedra de Microbiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 954, Piso 8, 1113 Buenos Aires, Argentina. Phone: 54 1 964 8285. Fax: 54 1 962 5341. E-mail: ggutkind@huemul.ffyb.uba.ar.

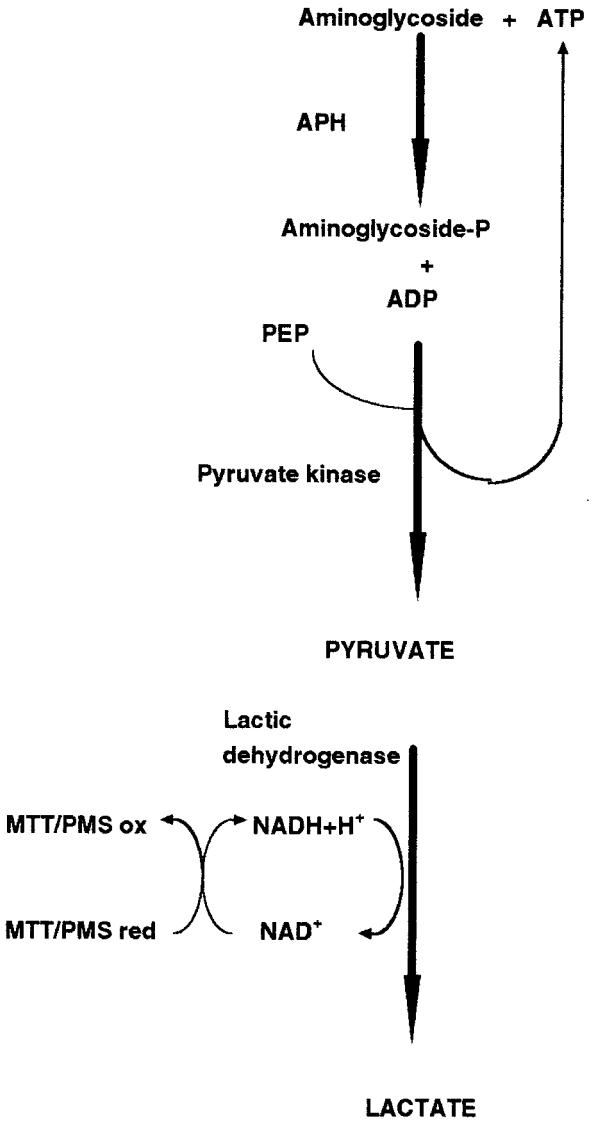

FIG. 1. Enzymatic reactions used for APH activity detection.

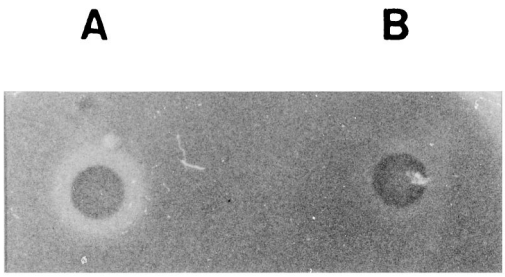

FIG. 2. Ten microliters of an extract of *E. coli* INM 7251 was poured onto disk A. The same amount of a nonresistant strain (*E. coli* ATCC 11105) was poured onto disk B. Phosphotransferase activity was detected as described in the text.

Argentina), collected by centrifugation, and resuspended in the same buffer. The suspension was ice cooled and disrupted with six 30-s bursts in a Vibracell VC 500 sonicator. Cell extracts were then clarified by centrifugation (15,000 × *g*, 30 min, at 4°C), and the supernatants were concentrated to 1 ml by ultrafiltration through an Amicon 10 membrane.

Enzymatic activity was confirmed by incubating extracts with a small amount of kanamycin and ATP and monitoring the loss of drug activity by a standard disk method (2).

Biochemical detection of phosphotransferase activity in the concentrated supernatants was carried out by a coupled colorimetric reaction as follows. The melted reaction medium thermostated to 42°C contained 0.9% agar, 10 mM Tris HCl, 10 mM magnesium acetate, and 25 mM ammonium chloride, pH 7.8. The following freshly prepared solutions were added to obtain the indicated final concentrations: NADH, 16 mM; pyruvate kinase, 76 IU; lactate dehydrogenase, 640 IU; phosphoenolpyruvate (32 mM; ATP, 8 mM (all purchased from Sigma Chemical Company), as well as kanamycin sulfate, gentamicin, or neomycin (0.5 to 2.3 mM) (kindly supplied by Armstrong) by adding concentrated solutions (4). For qualitative detection, the mixture was poured onto glass plates. Once it had solidified, a disk containing 10 μl of the concentrated supernatant or the same amount of an extract lacking phosphotransferase genes (negative control) was poured on and incubated for an hour in darkness at 37°C. A 10 mM thiazolyl blue (MTT) (Sigma)–1 mM phenazine methosulfate (PMS) (Sigma) water solution (7) was added and allowed to react at room temperature, until a blue color became evident (between 1 and 5 min). The enzymatic cascade is presented in Fig. 1. Analytical isoelectrofocusing of the concentrated supernatant was carried out with Pharmacia precast gel Inmobiline dry plates, pH 4.0 to 7.0 (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Localization of phosphotransferase activity was carried out by pouring the reaction mixture onto the gels, as described above.

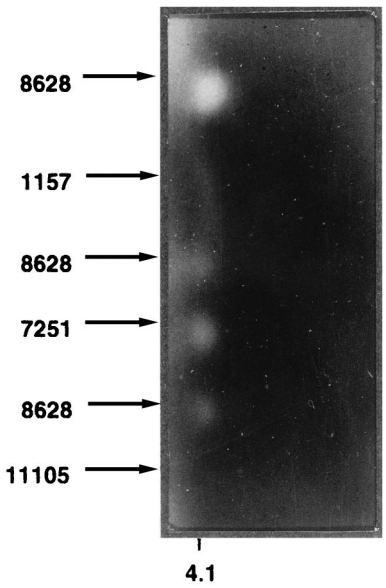

FIG. 3. Twenty microliters from each bacterial extract was electrofocused on Inmobiline dry plates (pH 4.0 to 7.0) at 2,000 V for 3.5 h. Phosphotransferase activity was detected as described in the text. Strains are indicated on the left.

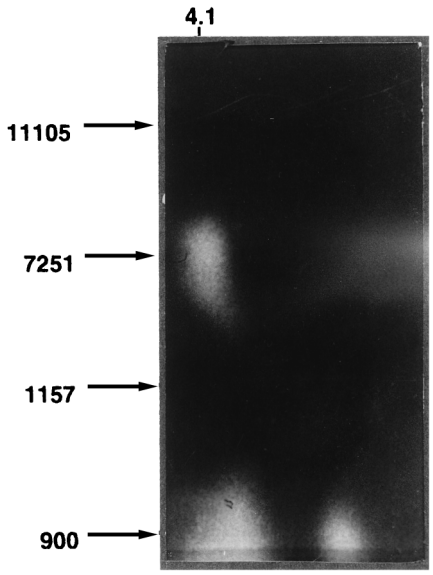

FIG. 4. Twenty microliters from each bacterial extract was electrofocused on Inmobiline dry plates (pH 4.0 to 7.0) at 2,000 V for 3.5 h. Phosphotransferase activity was detected as described in the text. Strains are indicated on the left.

RESULTS AND DISCUSSION

Figure 2 shows preliminary biochemical detection of phosphotransferase activity in crude extracts, using neomycin 1 mM as substrate. The same results were obtained when gentamicin was used at the same or higher concentration (data not shown).

As shown in Fig. 3, at an apparent pI of roughly 4.1, one active band appeared in two strains known to express a single phosphotransferase. A second band was observed at pI 4.4 in a strain harboring two such enzymes (Fig. 4). The band at 4.4 is in good agreement with the predicted pI value (8).

As expected from a reaction with an overall consumption of aminoglycoside, phosphoenolpyruvate, and MTT-PMS (reduced form) and production of aminoglycoside phosphate, lactate, and MTT-PMS (oxidized form), in the presence of the specific enzymatic system, only extracts with APHs were positive by this method. Although the enzymatic cascade theoretically might be less specific due to the action of other NADH or ATP-consuming or -producing systems, the absence of a suitable substrate in the reaction mixture precluded these reactions. Moreover, control extracts from strains lacking APH activity produced no decolorization. Furthermore, the widespread aminoglycoside acetyltransferase-producing strains in Argentina, most of which lack APH genes, also did not show any nonspecific reaction (data not shown).

Although only three APH-producing strains have been analyzed so far, the lack of nonspecific reactions and the detection of two active bands in the strain with two different APH genes suggest that this will be a useful method especially for the analysis of clinical isolates where phenotypic resistance cannot be attributed to any particular enzyme or isozyme. Hopefully, it may provide an easy and fast method for semiquantitative studies of APH activity in strains encoding the same (or different) information at the DNA level or presenting dissimilar expression. Lastly, our approach may also be used to

detect presumptively novel enzymes, when specific probes are not yet available.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from UBACYT and CONICET to G.O.G.

We thank A. Rossi for providing resistant strains previously characterized by DNA hybridization to aminoglycoside-modifying enzyme probes, which belonged to a group of local resistant strains analyzed by Shaw et al. (8) at the Schering-Plough Research Institute (Bloomfield, N.J.).

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