Inhibition of β-Galactosidase Biosynthesis in *Escherichia coli* by Tetracycline Residues in Milk

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Low levels of tetracyclines found as residues in milk inhibited the biosynthesis of β-galactosidase in *Escherichia coli*. To produce the same effect, other antibacterials had to occur in concentrations that were more than 10-fold higher. This relative selectivity was exploited for the development of a screening test for tetracyclines in milk based on a chemiluminometric assay of β-galactosidase. The method was validated with spiked samples of raw milk and applied to field samples contaminated with tetracyclines.

Many environmental toxicants inhibit the de novo biosynthesis of β-galactosidase in *Escherichia coli* (3, 5, 6, 7, 13), a feature that has found application in a commercial toxicity screening test (ToxiChromotest; Environmental Bio-Detection Products, Inc., Brampton, Ontario, Canada). This test involves exposure of an *E. coli* mutant strain to a toxic sample, followed by the colorimetric measurement of residual β-galactosidase activity (13).

As early as 1962 to 1963 it was reported that certain antibacterials, particularly chloramphenicol, inhibit the biosynthesis of inducible enzymes to a greater extent than they inhibit that of constitutive enzymes, presumably as a result of the enhanced production of a polynucleotide repressor at the operon level (19–22). However, while the observation itself proved correct, no evidence supporting the proposed mechanism was subsequently found. The question of whether the principle of inhibition of inducible-enzyme biosynthesis can be exploited in screening tests for residues of antibacterials arises. In milk and meat, the presence of such residues is undesirable because of the possible toxicity to consumers, the promotion of bacterial resistance, and the inhibition of food technological processes (4, 8, 10, 11, 16). The European Council has defined maximum residue limits (MRL) for a range of antibiotics and synthetic chemotherapeutics (1, 2). Commonly, the control of milk is performed at three levels, i.e., screening, group-specific testing, and confirmation. Screening tests are based mostly on the inhibition of growth of sensitive bacterial strains, as revealed by zone formation in a plate assay or the suppression of color change of a pH or redox indicator (9, 16).

The initial objective of our research was to examine to what extent inhibitors of bacterial protein synthesis were able to suppress the biosynthesis of β-galactosidase in *E. coli*. In view of a possible later application to milk, the ToxiChromotest was dismissed as a tool for this study because sample turbidity would disturb the colorimetric measurement. This interference could theoretically be eliminated by dilution, but dilution would cause the cell number of the test bacterium to fall below the detection limit of the colorimetric enzyme assay. We have previously developed a chemiluminometric assay of β-galactosidase in coliform bacteria as part of their detection in drinking water (12, 23). Reversing the concept of our approach, i.e., using *E. coli* as a test organism rather than as a target, laid the basis of an inhibition assay. Tetracyclines were unexpectedly found to inhibit the biosynthesis of β-galactosidase at concentrations much lower than those needed for other antibacterials, the difference being an order of magnitude or more. This relative selectivity was exploited for the development of a test, termed the tetracycline-galactosidase (TG) test, for screening of tetracycline residues in milk. The usefulness of this new method was judged in view of the inability of some existing microbial inhibitor tests to detect tetracyclines below the MRLs.

**Assay procedure.** Equal volumes of milk, a solution of iso-propyl-β-d-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) dissolved in 50 mM phosphate buffer (pH 6.5) at a concentration of 0.014%, and a suspension of *E. coli* ATCC 10536 in a minimal induction medium (MIM) (107 cells per ml) were combined and incubated for 90 min in a water bath at 37°C. The MIM contained the following ingredients (in grams per liter): KH2PO4, 1; MgSO4·7H2O, 0.05; NaCl, 5; sodium dodecyl sulfate (E. Merck AG, Darmstadt, Germany), 0.05; tryptone (Oxoid, Ltd., Basingstoke, United Kingdom), 1; and (NH4)2HPO4, 2 (pH 7.3).

The MIM was adapted from a low-level-nutrient medium designed previously (12, 23). This formulation was specifically designed to combine optimal chemiluminescence characteristics, i.e., low background and minimal quenching of light, with the capability to support maximal biosynthesis of β-galactosidase.

After the incubation, the mixture was diluted 1,000-fold with water to prevent light quenching due to sample turbidity. The β-galactosidase was assayed chemiluminometrically, as described previously (12, 23). The use of chemiluminesmetry was dictated by the need for a method with a substantially lower limit of detection than colorimetry to accommodate the low number of cells (104 CFU per ml).

To a 400-μl aliquot of the diluted mixture were added two 50-μl solutions, each made up of 250 mM sodium phosphate buffer (pH 6.5) supplemented with 10 mM MgCl2·6H2O containing either polymyxin B sulfate (100 μg ml⁻¹) (solution 1) or Galacton (50 μg ml⁻¹; Tropix, Bedford, Mass.) (solution 2). The solution was incubated for 45 min at 37°C and subsequently mixed with 100 μl of alkaline enhancer (1.6 mg ml⁻¹ in 0.5 M aqueous piperidine; Emerald [Tropix]) with the pump of
a luminometer (AutoLumat LB 953; EG & G Berthold, Bad Wildbad, Germany). Light emission, expressed as relative light units (RLUs), was measured over a 10-s period with a delay time of 2 s.

The decrease of enzyme activity relative to that of the control (hereafter referred to as percent inhibition control) was calculated by the following equation (13): percent inhibition = (1 – RLUs of the sample/RLUs of the control) × 100. The control contained all reagents as well as control milk but no antibiotic and was processed as the unknown. All results were the averages of results from three complete assays per sample. Assuming a probability of 95%, a sample was considered positive, i.e., presumed to contain tetracyclines, if the percent inhibition exceeded a threshold value of twice the relative standard deviation (RSD) of the mean response for control milk samples, i.e., milk devoid of antibiotics.

**Application to antibacterials in water.** Different antimicrobial agents were tested according to the above-described procedure but with water instead of milk. Concentrations ranged from 50 to 1,000 μg liter\(^{-1}\). A level of 100 μg of tetracycline hydrochloride per liter of water inhibited the activity of β-galactosidase by 42%. To produce a similar effect, other inhibitors of protein synthesis had to be present in five- to tenfold-higher concentrations, i.e., 500 μg liter\(^{-1}\) (chloramphenicol) and >1,000 μg liter\(^{-1}\) (macrolides and most aminoglycosides).

The observed difference in effects suggested a selectivity of the assay for low concentrations of tetracyclines relative to concentrations needed for other antibiotics.

**Application to antibacterials in milk.** Initially, the detection limits of tetracyclines in water could not be achieved in milk. When the procedure was applied to ultrahigh-temperature-treated (UHT) milk supplemented with tetracyclines at the MRL (100 μg kg\(^{-1}\) in the absence of a chelator, the inhibition rates \((n = 2)\) were 34 and 7% for tetracycline hydrochloride, 11 and 4% for oxytetracycline hydrochloride, and 12 and 0% for chlortetracycline hydrochloride. A possible explanation for the differences between results with water and milk was the complexity of tetracyclines in the latter with calcium ions. If so, the addition of a chelator would presumably eliminate the loss of activity in milk (17). In the presence of EDTA (12 mM as its disodium salt), the inhibition percentages increased significantly, i.e., to 57 and 28% for tetracycline hydrochloride, 45 and 50% for oxytetracycline hydrochloride, and 67 and 60% for chlortetracycline hydrochloride, thus supporting the hypothesis.

The replacement of EDTA by EGTA (12 mM; Sigma), which, unlike EDTA, does not chelate Mg\(^{2+}\) ions that act as cofactors for β-galactosidase, yielded similar results but with improved precision, as shown below. Macrolides, aminoglycosides, ampicillin, and sulfamethazine exhibited no inhibitory activity at levels up to 1,000 μg kg\(^{-1}\), whereas chloramphenicol gave 56% inhibition at this concentration but only 18% at 500 μg kg\(^{-1}\). Hence, the selectivity margin between tetracyclines and other antibacterials observed in water was confirmed in milk.

**Assay validation.** The following criteria were evaluated to demonstrate the validity of the assay: dose-response relationship, between-run precision on the dose-response curve, detection limit, repeatability, and specificity.

The occurrence of possible false negatives was assessed from the analysis of field samples that were found to be positive on the basis of established screening tests for tetracyclines. To exclude the possibility that raw milk, unlike UHT milk used for the initial method development, was itself inhibitory to β-galactosidase biosynthesis, the responses in both were compared over a period of 4 days with EDTA as the chelator.

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Based on the RSDs of 21 and 13%, the threshold values for a decision of positivity \((95\% \text{ probability})\) were set at 42% (EDTA) and 26% (EGTA). Only one false positive was found in both series \((28\% \text{ inhibition [EGTA] versus 47\% inhibition [EDTA]}\) in view of the superiority of EGTA as a chelator over EDTA in this assay, the former was used in all further experiments. This final procedure was termed the TG test.

Figure 1 shows dose-response curves for three tetracyclines (hydrochloride salts) added to control raw milk. Inhibition percentages for the three tetracyclines in concentrations up to 500 μg kg\(^{-1}\) were plotted against their concentrations.

Considering the above-threshold value of 26% inhibition, the detection limits can be extrapolated from these curves as approximately 70 μg kg\(^{-1}\) for tetracycline hydrochloride, 40 μg kg\(^{-1}\) for oxytetracycline hydrochloride, and 70 μg kg\(^{-1}\) for chlortetracycline hydrochloride. The corresponding values for the base forms were 65, 37, and 65 μg kg\(^{-1}\), respectively.

Oxytetracycline was further used to study the between-run precision of the dose-response curve (Fig. 2). The inhibition by oxytetracycline hydrochloride (TG test procedure) in concentrations ranging from 0 to 500 μg kg\(^{-1}\) was measured over four different days to yield a mean SD of 3.5%. When we assayed the UHT milk with EDTA over three different days (not shown), the following mean SDs were obtained: 4.1% inhibition (mean \(\pm \text{SD})\) (with RSD in parentheses) were 149 ± 32 RLUs \((21\%) \text{ (EDTA) and 167 }\pm 21 \text{ RLUs (13\%) (EGTA). The ranges were from 79 to 292 RLUs (EDTA) and from 121 to 214 (EGTA).}

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Assay specificity was reflected in the low rate of false positives with control milk and in the noninterference of other antibacterials in realistic concentrations; as examined with UHT milk, other antibiotics, including macrolides and amino-
glycosides, did not interfere at concentrations below 1,000 µg kg$^{-1}$ Chloramphenicol yielded 20% inhibition (i.e., below the threshold) at 500 µg kg$^{-1}$ and 48% inhibition at 1,000 µg kg$^{-1}$.

**Application to field samples.** “Naturally” contaminated milk samples were obtained from a healthy cow which had been given a single intravenous injection of 3 mg of oxytetracycline kg of body weight$^{-1}$ (Engzyme 10% dual dosage; Intertek International, Boxmeer, The Netherlands). Sampling was done before the administration of the drug and then every 12 h for 5 days. Screening for tetracyclines was performed by the TG test as well as three established tests, i.e., the Delvotest SP (Gist-brocades, Delft, The Netherlands), the Bacillus cereus test (18), and the LacTek tetracycline milk screening test (Idestek, Sunnyvale, Calif.). All commercial kits were used according to the manufacturers’ instructions. The Charm HVS-8100 tetracycline test (Charm Sciences, Malden, Mass.) was included for confirmation.

As shown in Table 1, the performance of the TG test was equivalent to that of the B. cereus and LacTek tetracycline tests but superior to that of the Delvotest SP. An advantage of the TG test over the B. cereus test is its higher speed. The latter test requires 16 to 24 h of incubation, whereas the total analysis time of the TG test is only 3 h, which is comparable to that of the Delvotest SP. False negatives were not found by the TG procedure.

A further field application came from the control of randomly selected farm milk samples that had been found to be positive for antibacterials on the basis of commercial screening methods (Charm HVS-8100 tetracycline test and the LacTek tetracycline milk screening test). Two samples that gave a positive response in the LacTek tetracycline test were likewise confirmed in our TG test. Conversely, eight samples containing aminoglycosides, sulfonamides, β-lactams, and chloramphenicol (Charm HVS-8100 results) remained negative in the TG test, which supports the specificity claim.

This new method has been welcomed by the Government Dairy Research Station as a valuable complement to the screening tests that are currently in use and that occasionally fail to detect tetracycline residues in milk.

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**REFERENCES**


15. Suhren, G. 1993. Experiences with an IDF-experimental study for the detection of beta-lactams, and chloramphenicol (Charm HVS-8100 results) remained negative in the TG test, which supports the specificity claim.

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