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Bioassay strain development for the analysis of bacitracin in bacitracin/chlortetracycline combinations

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A variant strain was developed from *Micrococcus luteus* ATCC 10240 for the purpose of bioassay analysis of bacitracin in the presence of chlortetracycline (CTC). Strain EN5 resulted from four sequential mutation steps, using quantitative resistance to CTC and retained bacitracin sensitivity as a selective criterion. Strain EN5 was tested for bioassay response, stability, and identity. The strain measured bacitracin activity with no interference from 40 μg ml^{-1} added CTC.

Keywords: antibiotics; bacitracin; chlortetracycline; bioassay; interference; strain development

Introduction

The use of an agar plate based bioasssay for antibiotic measurement was first developed by Heatley in 1944 [5]. Agar diffusion assay methods for antibiotics require the use of specific strains having sensitivity to the antibiotic measured. Assay interference can cause problems in zone development and reading [6]. Chlortetracycline (CTC) in particular has been more difficult to separate from virginiamycin [10] and bacitracin by chemical separation approaches. *Micrococcus luteus* ATCC 10240 is the primary test microorganism for the measurement of bacitracin in topical products and in feed samples. When measuring test mixtures of bacitracin and CTC, we found that the strain was also sensitive to CTC. Selective chemical inactivation of the CTC was considered, but we found that the published method required careful addition of the inactivating chemical under precisely controlled conditions to get a partially selective inactivation of the CTC. This report describes the first chlortetracycline-resistant test organism for quantitative bacitracin measurement when both compounds are present in a sample.

Materials and methods

Mutation and selection

Antibiotic medium #1 (Difco Laboratories, Detroit, MI, USA) agar plates containing CTC were prepared by adding appropriate amounts of stock solution to sterile molten agar at 50° C, then pouring petri plates to contain approximately 15 ml each. The stock solution consisted of 1000 μg ml^{-1} CTC (Sigma) in HPLC grade methanol acidified with a final concentration of 0.01 N HCl. Plates were stored at 4° C until used.

The CTC resistance levels were evaluated by streaking a small patch on a graded CTC-containing agar plate and incubating it overnight at 38° C. Gradient plates with different maximal amounts of CTC were also utilized to evaluate resistance levels of putative resistant isolates.

ATCC 10240 was grown on antibiotic #1 agar plates without CTC overnight at 38° C. The parent strain was previously found to be sensitive to CTC at concentrations of 0.15 μg ml^{-1}. Several swabs of culture were suspended in sterile peptone saline diluent (0.1 g L^{-1} soy peptone type II (Sigma) plus 0.09 g L^{-1} NaCl) to a density giving approximately 1×10^9 colony forming units per ml. Samples of 0.1 ml were evenly streaked to the surface of several agar plates. Plates containing 0, 0.2, 0.4 or 0.8 μg ml^{-1} CTC were exposed for 5, 10, and 15 s to UV with a germicidal light (General Electric G30T8, 30 watts, 235 NM, 20 in. distance), and were incubated in the dark for several days at 38° C. After three days, several isolated colonies grew on plates containing 0.2 or 0.4 μg ml^{-1} CTC. These were restreaked to graded CTC-containing plates and grown overnight to confirm the initial resistance level.

The candidate strains with the highest CTC-resistance levels were further screened for the ability to measure bacitracin. Both sensitivity to low bacitracin levels and sharp zone appearance were the criteria for selecting isolates for further work. The mutation procedures were based on those described by Carleton and Brown [3].

Bioassay tests

The strain was given several bioassay tests to demonstrate quantitative response to bacitracin with or without CTC in standard buffer solutions. Test points at different claim levels with or without CTC were also run to determine if the strain would be suitable for further work using complex matrices from commercial products.

Stock solutions of zinc bacitracin (AL Laboratories working standard, 68.9 U mg^{-1}) were prepared in 0.33 M phosphate buffer containing 53 ml L^{-1} reagent grade methanol and 947 ml L^{-1} deionized distilled water, final pH 6.5. Chlortetracycline hydrochloride (Sigma) was dissolved in reagent grade methanol containing 0.01 N HCl at a concentration of 1000 μg ml^{-1}. Bacitracin standard solutions at concentrations of 0.01, 0.015, 0.02, 0.025, 0.04, 0.08, and 0.16 U ml^{-1} were prepared in the same methanol phosphate buffer with and without 40 μg ml^{-1} CTC added from stock.

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The EN5 strain was propagated on antibiotic medium #1 (Difco). The cultures were then diluted and plated according to the standard procedure, with six 8-mm stainless steel sterile cylinders placed on the surface of the agar. Aliquots of 250 μl of standard solutions and buffer blanks were dosed in the cylinders.

Four independent trials were run for buffer curves. Each trial was set up with nine zones per concentration and thirty six reference points (0.04 U ml^{-1}). The plates were incubated for 18–20 h at 32–33° C and the zones measured using a video zone measurement system (Image Technology Co, Deer Park, NY, USA, model 30A100-5). The zone areas were computed based on the calibration from a defined area image. Linear regression formulas were calculated by the software, plotting $\log_{10}$ concentration versus corrected zone diameter averages as described elsewhere [12].

Test analyses of bacitracin solutions were then run using standard buffer curves run on four different days. The test solutions were prepared with defined levels of 0.015 U ml^{-1} and 0.025 U ml^{-1} bacitracin with or without additional spiked CTC (40 μg ml^{-1}). Bacitracin concentrations were calculated using the linear regression program described above. After the four trials, mean concentration, percent recovery, and percent relative standard deviation were calculated.

Stability and identity tests

The EN5 strain was further tested to demonstrate stability of the CTC resistance trait, and to identify the variant as derived from *Micrococcus luteus*. Twenty serial transfers were made on antibiotic #1 agar containing no CTC, incubating each culture overnight. The resistance level was then compared to a culture transferred once.

The identity of the mutant strain was evaluated by its carbohydrate utilization pattern compared to the parent, using Becton-Dickinson's MiniTek™ system (Becton-Dickinson Microbiology Systems, Cockeysville, MD, USA). A version of the MiniTek medium was prepared with the following formulation: NZ Amine A (Quest), 2.0 g; NaCl, 5.0 g; K_2HPO_4, 0.3 g; yeast extract (Difco), 1.0 g; deionized water, 1.0 L. Test tubes containing 0.9 ml of sterile medium were inoculated with two swabs of freshly grown culture, vortexed, and 0.5 ml of the inoculated broth was transferred to each well of a 24-well tissue culture plate containing the carbohydrate disks. Eight carbohydrates were selected for the utilization test. The plates were incubated at 38° C and were read after 4 and 7, and 24 h incubation.

Results

Mutation and selection

Table 1 summarizes the mutation sequence to obtain strain EN5, and the quantitative CTC resistance levels for each strain. The strains selected among the resistant isolates passed all screening criteria before additional bioassay tests or mutation. Figure 1 is a photograph of a screening test bioassay plate showing the large CTC interference zones present on lawns of *Micrococcus luteus* ATCC 10240, compared to EN5 lawns which had no interference under the same conditions.

Bioassay tests

Results of the buffer curve tests are presented in Figure 2. Strain EN5 responded in a linear manner to bacitracin, independent of the presence or absence of 40 μg ml^{-1} CTC in the buffer. The standard curve for bacitracin using strain EN5 was similar to that of the parent strain (data not shown), and the linear concentration range for measurement was the same. The results of bacitracin test point analyses are shown in Table 2. The average recoveries were within 9% of the test claim levels, which were at the lower end of the standard curve. Recoveries at the higher end of the curve gave similar results (data not shown). These results were below the acceptable tolerances of 15–30% recovery in the microbiological assay of various feeds and feed premixes [6, 12]. Experiments using feed matrix samples of bacitracin methylene disalicylate (BMD) at 10–250 g ton^{-1} concentrations in the presence and absence of CTC also gave similar results (data not shown).

Stability and identity tests

After twenty transfers in nonselective medium, the CTC resistance of strain EN5 was found to be unchanged from the original isolate slant. The breakthrough interference in a cylinder bioassay test for strain EN5 was detectable at 60 μg ml^{-1} of CTC in buffer for the original slant and after twenty transfers. The bacitracin standard buffer curves were identical as well. These tests indicated that the accumulated mutations were stable for strain EN5. It was found that the strain could be routinely transferred in nonselective medium prior to bioassay tests.

The MiniTek™ system tests gave identical carbohydrate patterns for strain EN5 and the parent strain. The key provided by Becton-Dickinson Microbiology Systems indicated a carbohydrate utilization pattern consistent with *Micrococcus luteus*. This test confirms the isogenic lineage of the EN5 strain from ATCC 10240, in addition to the yellow pigmentation that is characteristic of both strains.

Discussion

There are many examples of targeted resistance to get a selective response to only one antibiotic, eg *Micrococcus luteus* ATCC 10240A is resistant to dihydrostreptomycin and streptomycin, while ATCC 10240B is neomycin resistant [2, 4]. *Staphylococcus epidermidis* ATCC 12228 is used to assay neomycin, netilmicin, novobiocin and other antibiotics in preparations containing chlortetracycline [1, 4]. These variants are official test organisms for the assay of many antibiotic products [12]. Strains with targeted resistance developed through mutation or transduction have been used since the early 1950s [2, 9]. Tetracyclines interfere with protein synthesis by inhibiting the binding of aminoacyl-tRNA to ribosomes, while bacitracin acts by inhibition of cell membrane function and other mechanisms [13]. Tetracycline resistance may be caused by enhanced efflux mechanisms [7] or by ribosomal or other target modification [11].

The strain modification approach can be a useful method for the specific analysis of antibiotics in mixtures less amenable to chemical separation or selective inactivation. The results described in this study using the variant strain

Table 1 Strains developed for bacitracin measurement in the presence of chlortetracycline

Strain	Source	Agar plate chlortetracycline resistance (μg ml^{-1})	Bioassay zone diameter (50 μg ml^{-1} CTC) (mm)
ATCC 10240 (*Micrococcus luteus*)	ATCC	0.15	26
C7	UV mutant of ATCC 10240[a]	2.0	19
C7-4	UV mutant of C7	6.0	17
E9	EMS mutant of C7-4	20.0	13
EN5	ENU mutant of E9	45.0	9

[a] Mutagen abbreviations: UV, ultraviolet irradiation; EMS, ethyl methane sulfonate; ENU, *N*-nitroso-*N*-ethylurea

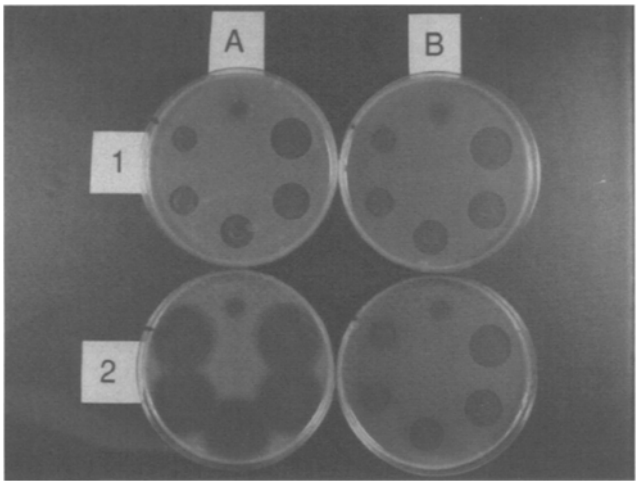

Figure 1 Response of the control and mutant strains to a screening test. All plates had the following concentrations of bacitracin (counter clockwise from top): 0, 0.01, 0.02, 0.04, 0.08, and 0.16 U ml^{-1}. Row 1 has no added CTC, row 2 has 40 μg ml^{-1} CTC. Column A lawns are *Micrococcus luteus* ATCC 10240, column B lawns are strain EN5

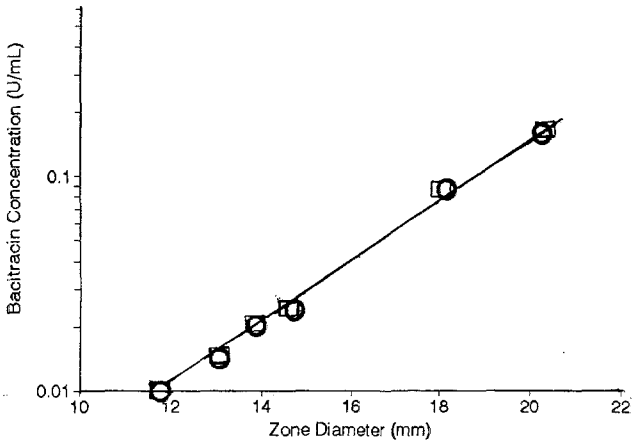

Figure 2 Average bacitracin response curve for strain EN5 using buffer standards. Open squares, no CTC added; open circles, 40 μg ml^{-1} CTC added. Regression analysis formula: log $y = 0.142 \times -3.668$. $r^2 = 0.9993$

Table 2 Analysis of bacitracin test solutions with and without spiked CTC using strain EN5

Day	Bacitracin (U ml^{-1})		Bacitracin (U ml^{-1}) + 40 μg ml^{-1} CTC	
	0.015	0.025	0.015	0.025
1	0.0160	0.0247	0.0133	0.0236
2	0.0183	0.0300	0.0152	0.0254
3	0.0157	0.0282	0.0185	0.0286
4	0.0139	0.0250	0.0151	0.0228
Mean	0.0160	0.0270	0.0155	0.0251
% Recovery	106.7	108.0	103.3	100.4
Std deviation	0.0018	0.0026	0.0022	0.0026
% RSD	11.25	9.63	14.19	10.36

demonstrate a significant improvement over the hypochlorite inactivation method [8].

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