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Identification and Characterization of Antimicrobial Activity in Two Yeast Genera

CARL A. BILINSKI, GINO INNAMORATO, AND GRAHAM G. STEWART*

Production Research Department, Labatt Brewing Company Limited, London, Ontario, Canada N6A 4M3

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A general screening test for the expression of antibacterial activity was performed on over 400 cultures belonging to 31 yeast genera. Of these cultures, only two, *Kluyveromyces thermotolerans* and *Kloeckera apiculata*, were found to produce zones of inhibition of bacterial growth on Diagnostic Sensitivity Test Agar medium supplemented with 0.002% methylene blue. Of nine bacteria used as test organisms, only *Lactobacillus plantarum* and *Bacillus megaterium* were inhibited. No antibacterial activity was evident against four gram-negative bacteria used in this study. Optimal activities were found to be expressed after yeasts were grown at pH 6. A requirement for cultivation in the presence of methylene blue added to culture media for the expression of apparent antibacterial activity was demonstrated.

Certain bacteria are well known to impart undesirable qualities to fermentation products. In particular, the metabolic activities of bacterial groups of concern to brewers, such as the lactic and acetic acid bacteria, can contribute an array of organic compounds that spoil the flavor and aroma of beer (8). As there is a constant need for strict microbial control to ensure consistent production of high-quality beers, the identification of yeast strains that secrete thermolabile antibacterial substances and the introduction of such characteristics into routinely used brewery strains would be of considerable value.

Although it is well known that penicillia, aspergilli, actinomycetes, and certain members of other microbial groups possess an ability to synthesize and secrete secondary metabolites that are pharmacologically active against the growth of other microorganisms (12), little attention has been given to *Saccharomyces* spp. as possible producers of substances with similar properties (5, 7, 9, 11). The killer (zymocidal) phenomenon is the only well-defined instance of anticontaminant activity in *Saccharomyces cerevisiae* (15), but the activity is directed against other yeasts and not against bacteria (16). The present study was performed in an effort to identify, from among over 400 *Saccharomyces* and non-*Saccharomyces* strains, those strains capable of secreting substances that inhibit the growth of beer-spoilage bacteria.

The yeast strains used in this study were obtained from the Labatt Yeast Culture Collection and from the Yeast Culture Collection of the University of Western Ontario, London, Ontario, Canada, and were as follows (number tested): *Aureobasidium pullulans* (2), *Candida* spp. (18), *Cryptococcus* spp. (5), *Debaromyces* spp. (2), *Endomyces magnusii* (2), *Endomycopsis* spp. (3), *Geotrichum penicillatum* (1), *Guilliermondella selenospora* (1), *Hansenula* spp. (9), *Kloeckera* spp. (2), *Kluyveromyces* spp. (7), *Lipomyces* spp. (2), *Metschnikowia pulcherrima* (1), *Nadsonia elongata* (1), *Pachysolen tannophilus* (1), *Pichia* spp. (6), *Prototheca zopfii* (1), *Rhodotorula* spp. (5), *Saccharomyces carlsbergensis* (52), *S. cerevisiae* (260), *Saccharomyces fragilis* (3), *Saccharomyces rosei* (2), *Saccharomyces rouxii* (2), *Schwanniomyces* spp. (2), *Schizosaccharomyces* spp. (4), *Sporobolomyces roseus* (1), *Sporopachydermia* spp. (2), *Torulopsis dattila* (1), *Trichosporon* spp. (4), *Wickerhamia fluorescens* (1), and *Zygosaccharomyces florentinus* (1). All yeast cultures were maintained on Wickerham (14) malt extract-yeast extract-peptone-glucose agar slant medium, which consisted of the following ingredients per liter of distilled water: malt extract, 3 g; yeast extract, 3 g; peptone, 5 g; glucose, 20 g; and Bacto-Agar (Difco Laboratories, Detroit, Mich.), 20 g.

Nine beer-spoilage bacteria obtained from the Labatt Bacterial Culture Collection were used as test organisms in this study and were as follows: *Bacillus megaterium*, *Bacillus subtilis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Pediococcus cerevisiae*, *Acetobacter aceti*, *Alcaligenes* sp., *Enterobacter* sp., and *Flavobacterium* sp. Stock cultures of these bacteria were maintained on slants of de Man-Rogosa-Sharpe agar medium (3; Oxoid Ltd., London, England). Bacterial cultures were incubated in accordance with the temperature and oxygen requirements specified in *Bergey's Manual of Determinative Bacteriology* (1).

In a preliminary screening of over 400 yeast strains for antibacterial activity, *L. plantarum* was used as the test organism, since it is one of the most commonly encountered brewery contaminants, and is tolerant of an acidic pH and ethanol (10). Yeast cells from 48-h malt extract-yeast extract-peptone-glucose agar slant cultures were transferred by means of sterile inoculation loops to the surface of Diagnostic Sensitivity Test Agar (Oxoid Ltd.) supplemented with 0.002% methylene blue (DST-MB) and seeded with *L. plantarum*. This medium contains the following ingredients per liter of deionized, glass-distilled water: proteose peptone (Oxoid L46), 10 g; veal infusion solids, 10 g; dextrose, 2 g; sodium chloride, 3 g; disodium phosphate, 2 g; sodium acetate, 1 g; adenine sulfate, 10 mg; guanine hydrochloride, 10 mg; uracil, 10 mg; xanthine, 10 mg; aneurine, 20 μg; and agar (Oxoid no. 1), 12 g. Bacterial lawns were prepared by overlaying petri dishes containing 15 ml of DST-MB medium (pH 6.2) with 10 ml of the same medium inoculated with 50 μl of an overnight nutrient broth (Oxoid Ltd.) culture supplemented with 1% glucose. After 24 h of incubation under aerobic conditions at 21°C to favor yeast growth and 48 h of incubation at 27°C under partial anaerobic conditions to facilitate bacterial growth, plates were examined for the presence of inhibition zones.

Of a total of 400 *Saccharomyces* and non-*Saccharomyces*

* Corresponding author.

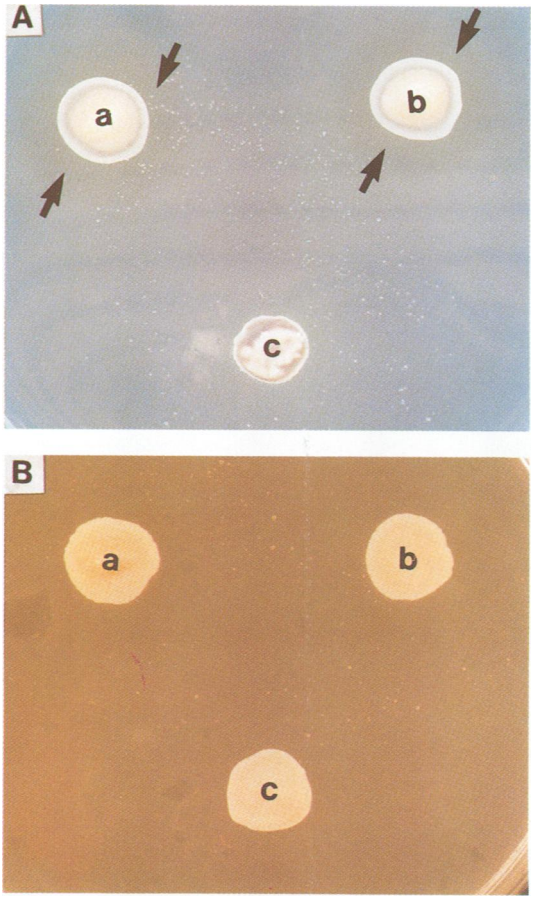

FIG. 1. (A) Inhibition halos (arrows) against *L. plantarum* produced by *K. thermotolerans* (a) and *Kloeckera apiculata* (b) cultivated on DST-MB medium. (B) Cultivation of *K. thermotolerans* (a) and *Kloeckera apiculata* (b) on Diagnostic Sensitivity Test Agar seeded with *L. plantarum* (no methylene blue). No inhibition zones developed. In both panels A and B a polyploid *S. cerevisiae* strain (c) was used as a negative control.

cultures tested for the ability to inhibit the growth of *L. plantarum*, only 2, *K. thermotolerans* and *Kloeckera apiculata*, were found to produce zones of growth inhibition on DST-MB medium (pH 6.2) (Fig. 1A). The optimal pH for the apparent expression of antibacterial activity was determined. Both yeasts were found to express maximal antibacterial activity when cultivated at initial pH values of 6 (Table 1). Unlike *Kloeckera apiculata*, *K. thermotolerans* produced no zone of inhibition on lawns of *L. plantarum* when cultivated at pH 6.5. Neither strain inhibited bacterial growth when cultivated in media adjusted to initial pH values of 4.5, 5.0, and 5.5. The lowering of the culture medium pH after yeast growth could not account for the inhibition of bacterial growth, since cultivation of the yeasts at these lower pH values did not result in zone formation. In addition, *L. plantarum* is well known to be tolerant of an acidic pH and ethanol (10). Since the optimal expression of antibacterial activity in both yeasts of interest occurred at pH 6.0, the experiments described henceforth, unless otherwise indicated, were performed with DST-MB medium at this pH value.

Of five gram-positive bacteria (*B. megaterium*, *B. subtilis*, *L. acidophilus*, *L. plantarum*, and *P. cerevisiae*) tested in plate assays for susceptibility to growth inhibition by *Kloeckera apiculata* and *K. thermotolerans*, only *B. megaterium* and *L. plantarum* were found to be susceptible (Fig. 1A). No antibacterial activity was evident against the four gram-negative bacteria (*A. aceti*, *Alcaligenes* sp., *Enterobacter* sp., and *Flavobacterium* sp.) used as assay organisms in this study. Thus, the antibacterial activities appeared to be not only gram-specific but also species specific. Moreover, they could not be attributed to acetic acid production (unpublished observation).

Kloeckera apiculata and *K. thermotolerans* were analyzed for the expression of antibacterial activity in DST-MB liquid medium. Volumes (50 ml) of DST-MB liquid medium in 250-ml Erlenmeyer flasks were inoculated with cells from 24-h malt extract-yeast extract-peptone-glucose agar slants at an initial density of 10^6/ml. DST-MB liquid medium was prepared by adding 0.002% methylene blue to filtered Diagnostic Sensitivity Test Agar (Oxoid Ltd.). Cell numbers were estimated with hemacytometer counting chambers. After 4 days of incubation at 27°C in a model G26 Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) operated at 150 rpm, cells were harvested by centrifugation for 15 min at 3,025 × *g* and 5°C. Supernatants were sterilized by filtration through Millex-GV syringe filters (0.22-μm pore diameters), and 10-μl volumes were spotted onto bacterial lawns. In both cases, cell-free spent culture filtrates displayed antibacterial activity, and the same spectrum as described above was found.

Thin-layer chromatographic (TLC) analysis of filter-sterilized spent culture filtrates orginating from 4-day DST-MB broth cultures of the two yeasts revealed the presence of distinct components (Table 2). These coincided with the R_f values of the methylene blue component of the spent broth culture media originating from *Kloeckera apiculata* and *K. thermotolerans* before $AlCl_3$ treatment and examination under UV illumination. There was no apparent migration of components originating from inoculated and uninoculated negative control DST-MB broth cultures. A solution of methylene blue alone was not detectable in the solvent system used, suggesting modification of the dye by the two yeasts into a form detectable in the solvent system.

To determine whether cultivation in the presence of methylene blue added to culture media was a prerequisite for the development of inhibition zones, we cultivated *Kloeckera apiculata* and *K. thermotolerans* in Diagnostic Sensitivity Test Agar with and without inclusion of the dye. Zones developed only when the dye was included in the culture medium (Fig. 1A and B), indicating a role for the dye

TABLE 1. Effect of culture medium pH on antibacterial activity[a]

Culture medium pH	Presence (+) or absence (−) of zones of bacterial growth inhibition with:	
	Kloeckera apiculata	*K. thermotolerans*
4.5	−	−
5.0	−	−
5.5	−	−
6.0	+ +	+ +
6.2	+	+
6.5	+	−

[a] Aliquots (5 μl) of cells from 48-h malt extract-yeast extract-peptone-glucose agar slant cultures were spotted at a density of 10^9 cells/ml onto DST-MB medium seeded with *L. plantarum*. Plates were incubated overnight at room temperature before being incubated for 2 days at 27°C under partial anaerobic conditions.

TABLE 2. TLC analysis of spent culture filtrates originating from *Kloeckera apiculata* and *K. thermotolerans*[a]

Source of filtrate	R_f value
Erythromycin (standard)	0.61
Uninoculated DST-MB broth[b]	No migration
Methylene blue (0.002%)	Not detectable
S. cerevisiae 1 (negative control)[b]	No migration
Kloeckera apiculata[b]	0.54[c]
K. thermotolerans[b]	0.55[c]

[a] TLC analyses were performed by the method of Wallhäusser (13). Volumes (5 μl) of filter-sterilized spent culture filtrates from 4-day DST-MB broth cultures were spotted onto Silica Gel G (firm 88; Merck & Co., Inc., Rahway, N.J.). The solvent system was a mixture of butanol, acetic acid, and deionized, glass-distilled water in a ratio of 2:1:1 by volume. Spots were visualized under UV illumination after treatment with 20% $AlCl_3$. Erythromycin appears as an intense black spot under UV illumination after $AlCl_3$ treatment.

[b] All DST-MB media contained 0.002% (wt/vol) methylene blue.

[c] Spots were blue under visible light before $AlCl_3$ treatment and examination under UV illumination. After $AlCl_3$ treatment and examination under UV illumination, spots were purple.

in the determination of antibacterial activity in *Kloeckera apiculata* and *K. thermotolerans*. In recent years, microbial substrate transformations have been used to derive novel and improved industrial fermentation products of interest (2, 4). It is conceivable that the expression of antibacterial activity by these two yeasts is not due to the secretion of secondary metabolites but instead involves the transformation of methylene blue into a pharmacologically active form. TLC analysis suggests modification of the dye into a form detectable in the solvent system used in this study (Table 2), and preliminary evidence from preparative TLC analyses suggests the involvement of chemically modified methylene blue. The possibility that the yeasts modified the nutritional environment in the presence of methylene blue so as to render the dye an antibacterial agent cannot, however, be excluded, since it is known that certain dyes can display bacteriostatic properties (12).

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