Isolation of Noninhibitory Strains of *Zymomonas mobilis*

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Wild-type *Zymomonas mobilis* strains inhibit the growth of *Escherichia coli*. We report the first isolation of noninhibitory strains, called *Zymomonas* inhibition negative (Zin−), after treatment with N-methyl-N'-nitro-N-nitrosoguanidine. A standardized soft-agar overlay procedure for detecting *E. coli* growth inhibition was also developed.

*Zymomonas mobilis* is a potentially useful organism for commercial biological ethanol production (2, 11, 14, 15). This bacterium performs consistently well in fermentation trials up to and including pilot plant scale (5, 13, 16, 18; Anonymous, Bio-hol: a Progress Report, Bio-hol Developments, Toronto, Ontario, Canada, 1983). Earlier literature reports by Goncalves de Lima and co-workers have suggested a possible role for *Z. mobilis* as a therapeutic agent (9, 10; for review, see reference 24). They described the ability of *Z. mobilis* to antagonize the growth of a wide variety of bacteria and fungi. The effects of inhibition by *Z. mobilis* are particularly evident during attempts to transfer functions from more catabolically diverse organisms, such as *Escherichia coli* and *Pseudomonas aeruginosa*, into *Z. mobilis* by conjugation (19, 22, 23). In our laboratory, studies on the physiological aspects of this inhibition have indicated that growth and conjugative ability of *E. coli* mating partners is inhibited in the presence of *Z. mobilis* (K. Leung and G. G. Khachatourians, Abstr. Annu. Meet. Can. Soc. Microbiol. 1983, AM14, p. 69; K. Leung, M.Sc. thesis, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, 1982). Antagonism of mating partners may have contributed to the widely fluctuating frequencies and strain-dependent responses reported for mating experiments with *Z. mobilis* (4, 6, 8, 21, 25; Leung, M.Sc. thesis). Characterization of growth inhibition by *Z. mobilis* has been recently expanded independently by ourselves (T. L. Haffie and G. G. Khachatourians, Abstr. Joint Annu. Meet. Genet. Soc. Am. and Genet. Soc. Can. 1984, Genetics 107. PT2: S43 and by Skotnicki and co-workers (19). The factor(s) responsible is a low-molecular-weight, agar-diffusible, heat- and protease-resistant, broad-spectrum activity which is produced by all strains tested (19).

A *Z. mobilis* strain lacking the ability to inhibit other microorganisms would be of obvious value. We describe in this paper the use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) to derive such isolates from a wild-type strain. The screening technique exploited the ability of wild-type *Z. mobilis* colonies to cause a halo of growth inhibition on a lawn of overlaid *E. coli* (T. L. Haffie, M.Sc. thesis, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, 1984). Isolates which did not cause a halo of growth inhibition were termed *Zymomonas* inhibition negative (Zin−).

The *Z. mobilis* strain used in this study was a subculture of strain CP4 (20) received from the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill. (NRRL B14023). This strain was designated GK646 in our culture collection. For experiments described here, *Z. mobilis* was cultured in YG liquid medium (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% glucose) or on plates solidified with 1.5% agar. Cultures were incubated at 30°C in static broth or on plates in a GasPak anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.). The indicator bacterium was a wild-type laboratory strain of *E. coli* K-12 (CA8000), designated GK19 in our laboratory culture collection. It was grown in Luria broth (5 g each of NaCl and yeast extract and 10 g of tryptone [Difco] per liter) at 37°C in a gyratory shaker bath.

To prepare cells for treatment with NTG, an overnight culture of GK646 was diluted 1:1 with fresh YG medium, dispensed in 1-ml aliquots into sterile 1.5-ml Eppendorf microcentrifuge tubes, and incubated for an additional 2 h. Cells were harvested by centrifugation (10 s at 10,000 × g; Beckman microcentrifuge B), washed with 1 ml of phosphate-buffered saline (8.5 g of NaCl, 0.3 g of KH2PO4, and 0.6 g of Na2HPO4 per liter), and resuspended in 1 ml of YG medium supplemented with NTG (100 μg/ml). Cells were exposed to the NTG for 2 h (0.01% survival) before being washed and resuspended in 1 ml of fresh YG medium. Incubation was continued for 1.5 h before cells were diluted with phosphate-buffered saline and plated on YG medium.

After 72 h of anaerobic incubation, the colonies were individually transferred with sterile toothpicks to duplicate YG plates (14 colonies per plate) and incubated aerobically at 30°C for 18 to 20 h. One of the duplicate plates was used for screening, whereas the other, a master plate, was stored at 4°C.

*E. coli* overlays were prepared by diluting an overnight culture of GK19 1:10 with phosphate-buffered saline (final optical density at 610 nm, 0.4; 5 × 10⁸ CFU/ml). A 2-ml aliquot of molten YG medium (1% agar, 50°C) was mixed with 1 ml of the diluted GK19 culture and immediately poured onto a screening plate. Overlaid plates were then incubated aerobically at 37°C. A clear zone was visible around *Z. mobilis* colonies within 3 h as the remainder of the overlaid lawn became turbid owing to the growth of *E. coli*. Halos were allowed to develop for at least 6 h before phenotypes were scored. Putative Zin− colonies were retrieved from the master plates, and additional independent tests were performed.

More quantitative data were obtained by standardization of the screening plate agar volume (15 ml), the *Z. mobilis* inoculum (10 μl of an overnight culture; optical density at 610 nm, 0.6; spotted onto YG plates), and preincubation time (20 h). In addition, colonies were screened in situ on spread plates by initially growing them anaerobically for 48 h.

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and then aerobically for 24 h before overlaying. Studies with modified screening protocols have demonstrated the necessity of a preincubation step. The time lag may be required to allow an inhibitory substance to accumulate to an effective concentration or may allow Z. mobilis to reach a particularly productive growth phase. Furthermore, we found that aerobic incubation enhanced inhibition and improved the screening sensitivity.

Colonies formed by cells which had survived NTG treatment showed considerable heterogeneity in size, ranging from <1 to 3 mm in diameter. Although we have observed small colonies on GK646 control plates, the variation in colony size was greatly increased by NTG treatment. About 10% of the 222 colonies screened after NTG treatment showed drastically reduced halo sizes relative to untreated controls. Standardized screening of surviving colonies showed halo sizes ranging from slight peripheral clearing to 2.5 cm in diameter for the GK646 control spots. Two strains (designated GK648 and GK649) had no halos after repeated testing (Fig. 1). These putative mutants grew more slowly than the GK646 parent but otherwise showed typical Z. mobilis glucose fermentation, colony and cellular morphology, and gram-negative staining characteristics.

We found no spontaneous wild-type revertant colonies on plates of GK648 (over 1,000 colonies screened) or GK649 (over 2,000 colonies screened). No spontaneously arising Zin strains were found among untreated GK646 colonies (over 500 colonies screened). It is noteworthy that treatment of strain GK646 with mitomycin C (25 μg/ml, 37°C, 48 h) or acidine orange (100 μg/ml, 37°C, 48 h) has so far failed to generate Zin strains (over 500 colonies screened).

Although the underlying genetic basis is unknown, the isolation of Zin strains is the first step in the genetic analysis of growth inhibition caused by Z. mobilis. These mutants and additional isolates could be used for the identification of structural and regulatory lesions, and we are hopeful that they will shed further light on the genetic and physiological nature of this intergeneric antagonism. Inhibition is the only genetic marker currently available for Zymomonas strains aside from antibiotic resistance and auxotrophy. Should it prove to be coded by a single gene or operon which contains unique restriction enzyme sites, it would be a good candidate for use as a genetic marker in the development of plasmid shuttle vectors. Inactivation of a plasmid-borne gene by cleavage with a restriction enzyme followed by insertion of passenger DNA is a well-established technique in current recombinant DNA technology (3). Insertional inactivation of a gene coding for inhibition could be readily detected in Z. mobilis by the screening procedure described here.

Intergeneric inhibition may have important consequences for the industrial application of Z. mobilis. The substrate range of known strains is restricted to glucose, fructose, and sucrose (24). This problem has prompted the study of simultaneous saccharification-fermentation systems involving cocultures of Z. mobilis with other microorganisms (1, 7, 17). If growth-inhibitory factors are produced during fermentation, Z. mobilis may have detrimental effects on a coculture system at the expense of productivity.

The biochemical nature of this antagonism is unknown. Primary metabolic products may be directly or indirectly responsible, or Z. mobilis may produce compounds similar to broad-spectrum antibiotics or bacteriocins. It is conceivable that the antagonistic factor(s) may jeopardize the proposed use of spent cells as a feed supplement (12) or present a health hazard in the handling of spent broth or the consumption of beverages fermented or contaminated by Z. mobilis.

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