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STUDIES ON A BACTERIOCIN-LIKE ACTIVITY PRODUCED BY PEDIOCOCCUS ACIDILACTICI EFFECTIVE AGAINST GRAM-POSITIVE ORGANISMS

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STUDIES ON A BACTERIOCIN-LIKE ACTIVITY PRODUCED BY *PEDIOCOCCUS ACIDILACTICI* EFFECTIVE AGAINST GRAM-POSITIVE ORGANISMS

By

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ABSTRACT

STUDIES ON A BACTERIOCIN-LIKE ACTIVITY PRODUCED BY

PEDIOCOCCUS ACIDILACTICI EFFECTIVE AGAINST

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Paul Walter Rueckert

Inhibitory activity by cultures of P. acidilactici, FBB-61, was studied in solid and liquid media using Lactobacillus plantarum, FBB-12, as the indicator organism. Conditions favoring optimal yield on solid media were determined, and production in liquid media was studied following the development of a standardized bioassay. The activity was found to be bactericidal, non-dialyzable across a semipermeable membrane, and stable to heat (100°C for 60 minutes) and freezing. The inhibitory agent is pronase-sensitive, and is destroyed by treatment with chloroform and ether. Certain of these data justify classification of the agent(s) responsible for the Pediococcus inhibitory activity as a bacteriocin. A 2X concentration of activity was achieved by the use of dialysis cultures, but all attempts to concentrate and/or purify the bacteriocin by techniques commonly used in the purification of proteins were unsuccessful. Several explanations for the apparent failure to achieve significant concentration in broth are discussed.

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INTRODUCTION

Lactic acid fermentations of brined cucumbers were studied extensively for many years in search of a means by which the frequent participation of undesirable organisms might be eliminated. Successful fermentations may involve up to five species of lactic acid bacteria. These are Lactobacillus plantarum, L. brevis, Pediococcus acidilactici (formerly P. cervisiae (2, 30)), Leuconostoc mesenteroides and Streptococcus faecalis (67). The latter two species are usually active only when the growth of P. acidilactici is delayed or absent (66, 67).

A study of pure culture fermentations of brined cucumbers (24) demonstrated that P. acidilactici inhibited the growth of L. plantarum. Simultaneous inoculation of the two organisms in cucumber brine resulted in a two day delay in the initiation of growth by the Lactobacillus. Both organisms initiated rapid growth in cucumber brines when inoculated individually. Further investigation revealed the lag before the growth of L. plantarum in mixed cultures may last a week or more, as indicated by acid production (23).

Certain strains of P. acidilactici are also inhibitory toward the growth of Staphylococcus aureus in broth cultures (34). However, the antagonism is relieved by the addition of yeast extract, tryptone, biotin, niacin or catalase to the medium. These investigators concluded that competition for vital nutrients, and the production of hydrogen peroxide contribute to the phenomenon.

Isolates of the Pediococcus genus from various sources were also tested for inhibitory activity against L. plantarum and other microorganisms by a seeded agar overlay technique (26). Two strains were found which consistently inhibited L. plantarum and other Pediococcus isolates, but not each other. Many gram-positive organisms were sensitive to the activities of the inhibitory strains, including L. mesenteroides 42, S. faecalis, Micrococcus luteus, S. aureus ATCC 10537 and Bacillus cereus T. None of the gram-negative bacteria or yeasts tested were suppressed. Addition of catalase to the growth medium had no effect on the inhibitory activity, eliminating hydrogen peroxide as the causative agent.

A great number of microbial toxins have been described which are responsible for the suppression of one population by another (1). In addition to hydrogen peroxide, other simple metabolic products including hydrogen sulfide, ammonia, nitrite, carbon dioxide, lactate, volatile fatty acids and ethanol are inhibitory in nature if they are allowed to accumulate to high levels in the surrounding medium (1). These differ from the "classical" antibiotics in that the latter are antagonistic to microbial growth when present at very low concentrations (76). Most antibiotics are organic compounds of low molecular weight and are typically the products of minor and secondary metabolic pathways (71).

Bacteriolytic or mycolytic enzymes are produced by some microorganisms, including certain species of Bacillus, Staphylococcus, Flavobacterium, Pseudomonas, Myxococcus, Streptomyces and Chalaropsis (1). Bacterial cultures are also lysed by bacteriophage infection, and some strains are lysed by defective bacteriophages; i.e., by phages

which are unable to complete a full growth cycle (1). Many investigators prefer to classify the latter entities with a group of substances loosely referred to as the bacteriocins (69). Bacteriocins are highly potent antimicrobial agents which usually have an essential, biologically active protein moiety and a bactericidal mode of action (78). However, exceptions to classical criteria based on the properties of colicins (bacteriocins produced by Escherichia coli) are encountered frequently (see Literature Review), and a precise definition of the term is not available.

Previous studies on the antimicrobial activities of P. acidilactici strains FBB-61 and L-7230 have been limited to those by Fleming et al (26) and Haines and Harmon (34), already described above. As this antagonism may be at least partially responsible for the rapid development of P. acidilactici prior to that of L. plantarum in natural fermentations of brined cucumbers and Spanish-type green olives (26), it is of interest to describe the phenomenon more fully. The present investigation was designed to determine the nature of the agent(s) responsible for the inhibitory activity; to develop a reproducible assay for quantitative analysis of cell-free supernatants; to describe the growth conditions which result in optimum yield; and to concentrate the activity for the future development of a purification scheme.

As mentioned above, the antimicrobial activity of P. acidilactici has been shown to be caused by something other than hydrogen peroxide (26), a common metabolic product of lactic acid bacteria. Results of the present study indicate that the activity is non-dialyzable across a semipermeable membrane and is bactericidal in nature. Agar cut from inhibitory zones, suspended in broth and spotted on indicator lawns

produced no plaques, which demonstrated the absence of self-replicating, infective entities (85). The agent is at least partially degraded by pronase, indicating the presence of a proteinaceous moiety on the molecule. These results led this investigator to believe the inhibitory activity of P. acidilactici is due to the release of a bacteriocin(s) into the growth medium. Thus, the Literature Review is confined to studies of bacteriocins, with emphasis on those bacteriocins produced by gram-positive organisms.

LITERATURE REVIEW

Definition

The study of bacteriocins began in 1925 when Gratia published his observations on a highly specific antibiotic produced by Escherichia coli V and effective against E. coli Ø (69). Gratia characterized the "antibiotic" as to its dialysibility, its stability to heat and chloroform, and found it was precipitated by acetone. With the discovery that this was but one of a group of similar antibiotics produced by the Enterobacteriaceae, Gratia and Fredericq suggested the agents be called "colicines" (69). Other organisms were also found to produce "colicine-like" antibiotics, and Jacob et al (62) proposed the more general term "bacteriocine" to include all highly specific antibacterial proteins which are effective mainly against strains of the producer species. Today, both terms are spelled without the final "e".

Mayr-Harting-et al (62) distinguish three overlapping periods in the history of bacteriocin studies. The first was largely descriptive, beginning with the Gratia publication and continuing until about 1950. Much of the technology for detection and assay were developed during this period. The second period began in the early 1950's and consisted of numerous studies on the genetics of bacteriocinogeny. These studies have shown most bacteriocinogenic factors are plasmid-borne (78), and many of these plasmids also determine the regulation of bacteriocin

synthesis, their release from the cells, and the host cell immunity. Certain bacteriocinogenic factors promote their own transfer on conjugation of the producer organisms with compatible recipient strains (46, 69, 81). A third field of investigation includes studies of the chemical nature of bacteriocins, their biosyntheses, and their modes of actions. Principle lesions usually occur in energy production, macromolecular synthesis, or membrane transport and permeability (78). However, bacteriocins of gram-positive bacteria may often promote bacteriolysis (3, 11, 39), sporostasis (54), and spheroplast formation (59).

A great deal of knowledge concerning the nature of bacteriocins has been obtained through these studies, and the field has been reviewed often (28, 37, 44, 62, 65, 68, 69). Until recently, the majority of the investigators have emphasized the bacteriocins of gram-negative bacteria, and especially the colicins. Thus, the classical definitions of bacteriocins were originally based on the characteristics of colicins, and generally included up to six criteria (78): a) a narrow inhibitory spectrum of activity centered about the homologous species; b) the presence of an essential, biologically active protein moiety; c) a bactericidal mode of action; d) attachment to specific cell receptors; e) plasmid-borne genetic determinants of bacteriocin production and of host cell bacteriocin immunity; f) production by lethal biosynthesis (i.e., commitment of the bacterium to produce a bacteriocin will ultimately lead to cell death).

However, the continued use of the term bacteriocin as defined above has been seriously questioned in a recent review on the bacteriocins of gram-positive bacteria (78). Relatively few bacteriocins, especially those produced by gram-positive organisms, are found to meet

the classical criteria which define the colicins. Thus, gram-positive bacteriocins often have activity spectra which extend to a number of organisms of different species and genera, the majority of these being gram-positive. A less solid host cell immunity is often associated with gram-positive bacteriocin production. As a result of such discrepancies, there is at present no universally accepted definition of the term bacteriocin. Personal preference often determines if a given antibacterial agent is labelled as a bacteriocin, a lytic enzyme, or a defective bacteriophage.

A number of substances which have been classified as bacteriocins have not been adequately characterized so as to justifiably place them under any classification at this time. Tagg et al (78) have urged restraint on the use of the term bacteriocin on incompletely defined antagonistic substances until they have at least been shown to have an essential protein moiety and bactericidal activity. Many investigators have already termed inhibitory agents under study as "bacteriocin-like" (7, 36, 44, 51) prior to further characterizations of the agents.

Demonstration of Bacteriocin Activity

The methods most commonly used to demonstrate bacteriocinogeny are performed on solid media and are generally referred to as the simultaneous and the deferred antagonism procedures (78). Both tests rely on the inhibition of an indicator organism caused by the bacteriocin released during the growth of a producer strain.

Simultaneous antagonism: This method involves the simultaneous growth of a producer strain and an indicator strain on or in a solid

medium. The technique most often used is to spot a small volume of producer culture onto the surface of a plate that has been freshly seeded with the indicator organism so as to yield confluent growth upon incubation (80). A zone of inhibition appears around the growth of the bacteriocinogenic culture. The procedure has been modified for facultative anaerobes by inoculating the plate as a stab into the agar rather than as a surface spot (5, 18). Another variation employes the use of wells in freshly seeded agar which are filled with agar containing the producer strain (72). Some investigators have sprayed the indicator culture onto the plate after the producer strain had been inoculated (43, 49). Each of these methods relies upon the early production of the bacteriocin and upon its diffusion through solid media.

Deferred antagonism: This procedure allows for the accumulation of the inhibitor prior to testing. The producer organism is grown on agar medium for a designated period of time and then killed by exposure to chloroform or heat (15). An agar overlay freshly seeded with the indicator strain is poured onto the surface of the plate, and the plate is incubated for a suitable time. Alternatively, the indicator strain may be streaked across the killed cells of the producer organism (62). Deferred antagonism techniques have often been proven to be more sensitive than simultaneous antagonism procedures, and allow independent variation of time and conditions of incubation of the producer and indicator cultures (78). It is imperative that the investigator determines the stability of the activity against the method of sterilization used. Bacteriocins vary widely in their heat stability (69), and at least one has been found to be chloroform-labile (9).

Either method of detection is dependent upon both the production of

the bacteriocin and its diffusion through the medium employed (62). The composition of the medium has been shown to directly or indirectly affect the diffusion of colicin (63) and the sensitivity of an indicator strain to a bacteriocin of Streptococcus mutans (70). It has been suggested (62) that a wide variety of conditions be applied in a search for bacteriocinogenic properties of microorganisms.

Assays for Quantitation of Bacteriocin Activities

There are no specific chemical or physical reactions for the assays of bacteriocin activity, which has led to the common use of microbiological assays for the quantitation of these compounds (62). Jacob et al (62) suggest that while such assays often have the disadvantages of low precision, lengthy incubation times, and difficulties in comparing yields of different types of bacteriocin, the assays do have a high specificity and a high sensitivity. The titers of bacteriocin assays are usually defined as the reciprocal of the highest dilution to cause inhibition of an indicator organism under standardized conditions, and is expressed as Arbitrary Units (A.U.) per ml of sample. It is necessary to consider if non-specific inhibitory activity is contributing to the titer and to eliminate viable producer cells from the sample before performing the assay (62). The latter is commonly accomplished by heat (13, 16, 79, 80); filtration (6, 11); or chloroform (20, 28, 32). The investigator must make certain that the method of sterilization does not reduce the titer either by inactivation of the bacteriocin or by interference of the assay.

Critical dilution method: This simple method is the most commonly used for both qualitative detection and for quantitative assay of a number of bacteriocins (62). Serial dilutions (usually two-fold) are made of the sample and uniform drops of each dilution are transferred to the surface of plates seeded with an appropriate indicator organism. The plates are incubated for a designated time interval and the degree of inhibition due to each drop is examined. The end-point is usually considered to be the highest dilution which resulted in complete inhibition, i.e., no visible growth, of the indicator organism. The titer (A.U./ml) is the reciprocal of this dilution. All conditions of the assay must be carefully standardized, including the size of the drop, the indicator inoculum size and the incubation time. The obvious limitation of this assay is the subjectivity in determining the end-point, but the simplicity of the procedure usually outweighs the inherent lack of precision (62). The method is highly reproducible if care is taken to maintain the appropriate set of defined conditions (32).

Survivor count method: This method assumes that under certain idealized conditions, the reaction of bacteriocin with a large excess of indicator cells will result in a distribution of bacteriocin particles among the cells according to the Poisson Distribution (62). The bacteriocin concentration is expressed in Lethal Units (L.U.), i.e., the minimum amount of bacteriocin which will kill a single sensitive indicator cell, and is derived from the equation:

$$L_o = -N_1 \times \ln (N_2/N_1)$$

where L_o = L.U. per ml of sample; N_1 = viable cells per ml at time of mixing; N_2 = viable cells at end of experiment. An indicator suspension is equilibrated at the temperature of reaction, after which a sample is

withdrawn for viable counting; this gives an estimate for N_1. A sample containing bacteriocin is added in known proportion to the cell suspension and mixed. After an adsorption period, a sample is again withdrawn to determine viable counts (N_2). The time allowed for adsorption and the temperature at which reaction mixtures are incubated have varied with the organism and bacteriocin under study. Reaction conditions have included 10 minutes at 36°C (62); 20 minutes at 37°C (40); 45 minutes at 36°C, followed by 1 - 2 hours in the refrigerator (61); 45 minutes at 37°C (56); and 2 hours at 0°C (56). The assay is reasonably precise and highly sensitive if the counts are made with a sufficient degree of replication (62).

Modified survivor count method: This assay substitutes the measurement of light-absorbing properties of the indicator suspension for the determination of viable counts in the above bioassay, thus increasing its rapidity. The procedure described by Jetten et al (49) is performed by mixing serial dilutions of bacteriocin in sterile tubes with tryptcase soy broth and 10^7 bacteria from an exponential phase culture of the indicator strain. The tubes are incubated at 37°C for 4 hours and the optical density is measured at 600 nm. A control tube containing no bacteriocin is also inoculated, from which the optical density corresponding to 100% survival is determined. An S-shaped curve results when absorbance is plotted arithmetically against the dilution of bacteriocin. The titer (A.U./ml) is determined from the graph as the reciprocal of the dilution yielding 50% increase in absorbance.

A drawback of this assay might be the contribution to the light-absorbancy of the suspension made by the cells killed by the bacteriocin (62). Several variations to the assay have been described which are

attempts to eliminate this problem. One involves the use of the redox indicator 2,3,5-triphenyl tetrazolium chloride which is reduced to its red-colored formazan intracellularly by metabolizing cells (73). Another depends upon the measurement of ultraviolet-absorbing material released from killed cells (52).

Production of Bacteriocin

Conditions of culture: The ability to produce a bacteriocin is usually genetically stable in bacteriocinogenic strains, but synthesis does not occur all the time or under all conditions (68). It is important to identify the optimum medium and conditions of incubation prior to the development of a purification scheme.

Some bacteriocins of gram-negative bacteria (55, 68) and many bacteriocins of gram-positive organisms (8, 29, 36, 41, 53, 74, 77, 82, 84) are produced at significant levels only on solid media. Increasing the viscosity of liquid media by the addition of agar, dextran, glycerol or starch has been shown in one study to increase the yield of bacteriocin (78). The yield of staphylococcin 1580 may be up to twenty times greater in a semisolid medium than in the corresponding liquid medium (49).

In general, complex media appear to support higher levels of bacteriocin than do simple media (62). However, large fluctuations in yield have been noted with different batches of these types of media (25, 79). Tagg et al (79) recovered streptococcin A from cultures in tryptic soy broth and Difco Todd-Hewitt broth, but not from Oxoid Todd-Hewitt broth, trypticase soy broth, tryptose phosphate broth or brain-heart infusion. Similarly, Rodgers (70) found that some strains of

S. mutans produced bacteriocin in Oxoid brain-heart infusion agar but not in BBL brain-heart infusion agar, while the reverse was true for other strains.

The primary carbon source and various supplements to the growth media have also been shown to affect bacteriocin production. Glucose inhibits the synthesis of colicin K (60), streptococcin B-74628 (77) and staphylococcin 462 (35), but stimulates the production of streptococcin A-FF22 (80). The addition of mannitol enhances the yield of staphylococcin 462 but decreases that of staphylococcin 414 (35). Yeast extract enhances bacteriocin production of certain strains of S. mutans (70) and group B streptococci (77), but represses the synthesis of bacteriocin from B. stearothermophilus strain NU-10 (85). Production of bacteriocin by Clostridium butyricum NCIB 7423 in a semi-defined medium is directly proportional to the amount of casein hydrolysate added (11).

The pH of the growth medium may be critical for optimal production of many bacteriocins. Goebel et al (31) found it essential for E. coli K 235 L O cultures to be maintained at pH 7.0 for maximum recovery of colicin K. Production of streptococcin A-FF22 on Todd-Hewitt agar was found to be best at pH 6.0 to 6.5, and no bacteriocin was detected at pH 7.5 or above (80). Production of staphylococcin 1580 in trypticase soy broth is optimum between pH 6.5 and 8.0 (49).

Temperature of incubation is also important for optimum yield. Generally, the temperature optimal for growth of the organism will result in the maximum production of bacteriocin (78). Growth at elevated temperatures often inhibits bacteriocin synthesis completely (14, 77), and may lead to an irreversible loss of activity (14, 78). The latter

phenomenon is usally associated with the loss of the bacteriocinogenic factor (78).

Aeration of cultures greatly increases the yield of certain staphylococcal bacteriocins (13, 49), and no activity was found in anaerobically grown S. epidermidis cultures (49). Conversely, production of bacteriocin by group A streptococcus strain FF22 was completely suppressed if the cultures were shaken (79).

Inhibitors and inactivators: Many bacteriocinogenic cultures excrete substances which are antagonistic to the bacteriocins they produce. Some mutants of E. coli release a specific inhibitor of colicin B into the growth media (33). Release of a specific teichoic acid in the latter stages of the growth cycle of S. faecalis subsp. zymogenes strain X14 results in the disappearance of bacteriocin activity in the cultures (17). Dialysis of culture supernatants containing staphylococcin 1580 greatly increases bacteriocin activity, which has led investigators to believe that a low molecular weight inhibitor may be present in the preparation (49). Protease produced by the bacteriocinogenic strain may inactivate bacteriocins of Serratia marcescens (27), Clostridium botulinum (22) and group A streptococci (79). Some of these proteases are denatured by boiling, thus protecting those bacteriocins which are heat stable (22, 79). Bacteriocin inactivators may be responsible for low activities under conditions which are conducive to their production and release into the growth media.

Production as a function of the stage of growth: Maximum bacteriocin yields may occur at different phases of the growth cycle, and the timing of harvest must then be determined empirically for each organism and set of conditions. Streptocin STH_1 production is optimal during

exponential phase, but levels drop sharply before the culture enters stationary phase (75). Staphylococcin C55 levels increase throughout the exponential phase, reaching a maximum between 24 and 48 hours of growth, and then gradually decline (16). Butyricin 7423 is released during late exponential phase, while perfringocin 11105 only appears at the onset of stationary phase (11). Some strains of Cornebacterium diphtheriae release bacteriocin continuously whereas others appear to produce it in bursts (78). Several studies have reported substantial losses of bacteriocin levels on prolonged incubation of cultures, which may be due to enzymatic degradation, appearance of specific inactivators, or readsorption to the producer cells (78).

Induction: Bacteriocin yields may often be increased many-fold by induction in a manner analogous to prophage induction. The most commonly employed methods of induction are treatments with ultraviolet irradiation or with mitomycin C (78). Other procedures that have been used include treatment with nitrogen-mustard, hydrogen peroxide, N-methyl-N'-nitro-N-nitrosoguanidine and cold shock treatment (62). However, cases are known where attempts to induce bacteriocin production have reduced the yield (13, 58). While a majority of gram-negative bacteriocins are reported to be inducible, a significantly smaller proportion of gram-positive bacteriocins have been successfully induced by the procedures mentioned above (78).

Properties of Bacteriocins

Chemical composition: Although bacteriocins are a chemically diverse group of substances, the presence of an essential protein component

provides a unifying property. Some bacteriocins of gram-positive bacteria appear to be simple proteins (38, 39), while others, including certain staphylococcal (29, 35), clostridial (78), and lactobacillal (19, 83) bacteriocins seem to be quite complex with lipid and carbohydrate components in addition to protein. The composition of staphylococcin 414 has been likened to that of the staphylococcal cell membrane (29).

Physical properties: The bacteriocins range in size from molecular weights of 8,000 to complex defective phage particles with molecular weights in excess of 10^6. Certain preparations of staphylococcin (29) and megacin C_x-337 (21) were found to contain ring-like structures of diameters 1.0 to 6.4 pm similar to membrane vessicles. Many bacteriocins produced by gram-positive organisms are believed to exist in two or more distinct physical forms (11, 22, 29, 35, 49, 75, 77, 83). The different molecular components of some of these bacteriocins appear to exist in equilibrium, with the degree of association and dissociation being influenced by pH and the ionic strength of the preparation (78).

Stability: The stability of a bacteriocin preparation is an important factor to consider during the development of a purification scheme. Increased purification often results in decreased stability (22, 57, 77, 79). Bovine serum albumen has been used successfully by some investigators to prevent excessive inactivation of the bacteriocin (29, 64).

The stability of bacteriocins to pH extremes is extremely varied, but most of these compounds seem considerably more tolerant of acid than alkaline pH treatments (78). Thus, megacin A-216 and streptococcin A-FF22 are both stable at pH 2 - 7 (45, 79), and staphylococcin 1580 is

stable at pH 3.5 - 8.5 (47). Both butyricin 7423 and perfringocin 11105 are stable from pH 2 - 12 (11), and boticin E-S5 is stable from pH 1.1 - 9.5 (22). Other bacteriocins have a much narrower range of pH values over which they are stable. Thus, boticin P is stable only at pH 6.5 - 7.5 (54).

Heat stability of bacteriocins is more difficult to define, as this property is dependent upon the state of purification, pH ionic strength and the presence or absence of protective molecules (78). The range of thermostability among the bacteriocins is wide. Boticin P is sensitive to a treatment of 60°C for 30 minutes (54), and streptocin STH_1 is inactivated in 10 minutes at this temperature (75). However, closticin A is resistant to a 30 minute treatment at 100°C (42), and lactocin LP27 can withstand 60 minutes at this temperature (83). Staphylococcin 1580 is still bioactive after a 15 minute exposure to 120°C (49).

Isolation and Purification

Isolation: Cells and cell debris of the producer organism are often removed by centrifugation or membrane filtration, though loss of activity sometimes occurs with the latter method (62). However, many bacteriocins are predominantly cell-bound, in which case separation from the cell mass may be made by a variety of physical, chemical, or enzymatic means (78). These include homogenization of the cells, mechanical disruption, elution with urea and sodium chloride, acid extraction, heat treatment and trypsin-lysozyme treatment. Alternatively, the activity may be diffused into a solid medium, which is usually

extracted with a suitable eluant or via means of a "freeze-thaw" technique (55).

Purification: Where the initial source of bacteriocin is insufficiently concentrated, large volumes of material are required and an initial concentration step is necessary (62). Among the successful methods of concentration are evaporation in a rotary evaporator at 40°C, lyophilization, and ultrafiltration (62). The purification process for non-dialyzable bacteriocins may be aided by the use of diffusates of the nutrient medium (16, 19, 49). The starting material is then free of all high molecular weight, non-bacterial substances, which simplifies subsequent operations.

Following concentration, the impure material is subjected to a suitable combination of purification methods, including fractional precipitation, fractional absorption, column chromatography (ion exchange and/or molecular exclusion), centrifugation, electrophoresis, and isoelectric focusing (62). The choice as to the combination of these methods and the order in which they are employed is largely empircal. A common problem in bacteriocin purification is the loss of activity as purification progresses (78). Thus, the specific activity (units of bacteriocin per milligram protein) is usually monitored at each step, and the procedure is modified where possible where there is excess loss of activity. The purified bacteriocin preparation is tested for homogeneity using more than one criterion. Common techniques used include gel electrophoresis, ultracentrifugal analysis, and/or immunological methods (62).

MATERIALS AND METHODS

Cultures and growth conditions: The producer organism used throughout this study was P. acidilactici FBB-61 (formerly P. cerevisiae FBB-61 (2, 30)). The indicator organism was L. plantarum FBB-12. Both organisms are gram-positive, homofermentative lactic acid bacteria and were isolated from commercial cucumber brines. Stock cultures were maintained in stabs in LBS agar (BBL), and were transferred regularly at 3-month intervals.

The basal growth medium employed was trypticase soy broth (BBL) buffered with potassium phosphate (pH 7.2) to 0.1 M (TSB). The phosphate was added aseptically to the medium after autoclaving to prevent the formation of precipitate. Additional glucose was added aseptically after autoclaving to a concentration of 1% unless otherwise indicated. All cultures were incubated at 30°C without shaking.

Agar overlay technique: This procedure was used most often to study inhibitory activity on solid media. The effector organism was grown on the surface of trypticase soy agar (TSA) as colonies or in streaks for an appropriate time interval. The plate surfaces were sterilized by a 3-minute exposure to vapors from 0.3 ml of chloroform on filter paper strips (2.0 cm square). The petri plates were then ventilated briefly and overlayed with 4 ml of soft agar medium freshly seeded with L. plantarum (10^6 cells/ml). After 18 - 24 h of incubation at 30°C, the plates were examined for zones of inhibition in the agar

overlays. The width of each zone was measured from the edge of the colony to the outermost point of inhibiton (clearing).

Standard assay for bacteriocin activity: Bacteriocin activity in broth media was quantitated by use of an assay adapted from that of Reeves (68). Cells were removed from broth cultures of the producer organism by centrifugation (10^3 x g, 4°C). Two-fold serial dilutions were made of the culture supernatant in fresh TSB. Each tube (5 ml volume) was then placed in a boiling water bath for 6 min to destroy any remaining producer cells. After cooling to room temperature, each dilution was inoculated (1%) with late log-phase L. plantarum cells diluted to an $O.D._{600}$ of 0.1. All tubes were incubated at 30°C for 16 h, at which time the optical densities were read at 600 nm. The reciprocal of the dilution yielding a 50% increase in optical density over that of the undiluted sample was taken to be the titer expressed as Arbitrary Units (A.U.) per ml.

RESULTS

Demonstration of Activity

Solid media: Growth of L. plantarum was consistently inhibited on TSA either by the simultaneous growth of P. acidilactici or by a product(s) of the latter organism remaining in agar after killing or removing the viable cells. Simultaneous antagonism was demonstrated by inoculating the surface of agar media with log-phase cultures of the two organisms in perpendicular streaks and allowing the plates to incubate at 30°C for 4 to 5 days. The development of L. plantarum was ordinarily inhibited within zones of 1 - 2 mm from the edge of the pediococcal growth.

Deferred antagonism was demonstrated in two ways. First, the agar overlay technique (Materials and Methods) consistently resulted in inhibitory zones in the soft agar of 4 - 6 mm from the producer culture. However, these zones were sometimes hazy, and their clarity was inversely related to both the amount of chloroform used in sterilization and to the duration of exposure to the vapors. Plates exposed to vapors from 0.3 ml chloroform for 5 min developed zones of inhibition as usual, but plates prepared in an identical manner but exposed to the vapors for 10 min or more were entirely free from inhibitory zones. These data indicate the bacteriocin activity is chloroform labile. Much wider zones were obtained by complete removal of producer cells.

Colonies on the agar surface were cut out using a cork bore of appropriate size, and the resulting plug of agar was removed with the tip of a spatula. A 10-fold dilution of exponential-phase indicator cells was streaked up to the edge of the hole in the agar, and the plate was incubated overnight. A clearly defined zone of inhibition developed which extended up to 20 mm from the edge of the hole (Plate 1).

Liquid media: Inhibitory activity was demonstrated in broth cultures by following viable counts in a mixed species inoculation of P. acidilactici and L. plantarum. Each organism was inoculated at approximately 10^5 cells per ml in TSB. Total viable counts were determined by plating samples on TSA. Viable counts of L. plantarum were determined on TSA containing 0.2% sucrose in place of glucose. The Pediococcus culture does not utilize sucrose and grows much slower than the Lactobacillus on this medium. Viable counts of P. acidilactici were obtained by taking the difference between the two counts. The results are presented in Fig. 1. Early development of the L. plantarum culture was followed by a rapid decline of viable counts at approximately 18 h, while P. acidilactici appeared to develop normally. The L. plantarum population remained below 10^2 cells/ml for several days. This organism made a much more rapid comeback against the pediococci in an earlier study in which the investigators used cucumber juice broth for the growth medium (24).

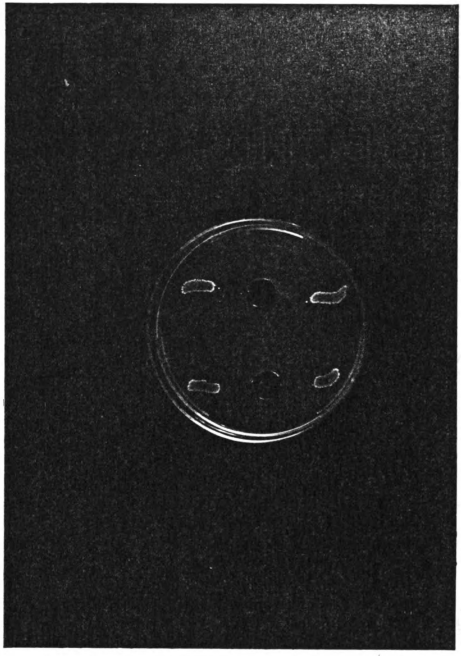

Plate 1. Deferred antagonism of L. plantarum by previous growth of P. acidilactici on TSA.

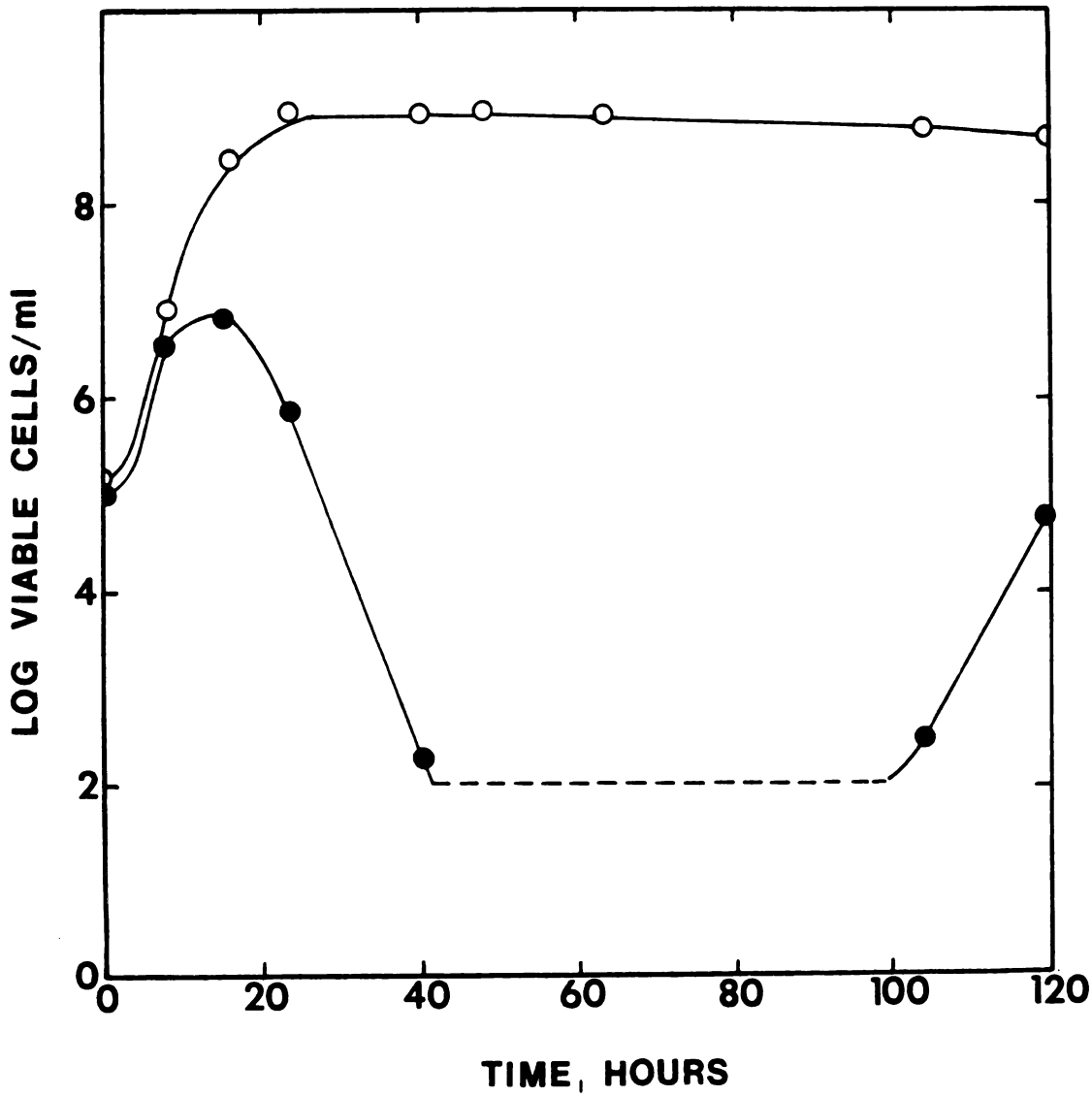

Figure 1. Mixed culture growth curves of P. acidilactici (o) and L. plantarum (•) in TSB.

Production

Solid media: Bacteriocin production on solid media was studied as a function of carbohydrate addition, pH, inoculum size and incubation time and temperature. Activity was determined using the agar overlay technique and measuring the widths of the resulting zones of inhibition. Substitutions of maltose, mannose, fructose and yeast extract for glucose in TSA had little or no effect on the size of the inhibitory zones. Similarly, supplementing a 1% glucose medium with carbohydrates and yeast extract at concentrations of 1% and 2% had no effect on the expression of activity. The inhibition of indicator growth was most pronounced at neutral pH values, and the activity decreased as the inoculum size of the effector strain was decreased (Table 1). Growth of the effector organism at 25°C resulted in smaller zones of inhibition than those produced at 30°C or 37°C. The zones reached their maximum diameters after 5 days of incubation (Table 2).

Liquid media: Prior to studying the production of the bacteriocin in broth media, it was necessary to develop a suitable assay and an appropriate method for sterilization of the spent broth. The critical dilution assay (see Literature Review) was unsatisfactory for this study, as no activity was observed when culture supernatant was spotted on either a lawn of indicator cells or on overlays seeded with the indicator strain. Thus, an assay similar to the Modified Survival Count Assay described by Jetten et al (49) was developed and used throughout this study. This assay is described in detail in the Materials and Methods section. Plastic caps were used on test tubes after it was observed that a substance toxic to L. plantarum was extracted from

Table 1. Inhibitory activity of P. acidilactici on TSA as a function of inoculum size

Cells/ml[a]	Width of inhibitory zone, mm[b]
10^8	4.0
10^7	3.0
10^6	3.0
10^5	3.0
10^4	2.0
10^3	1.0

[a] Log-phase P. acidilactici cells in TSB were harvested, washed once in 0.1 M potassium phosphate (pH 7.0) and diluted to approximate cell concentrations listed above. Aliquots of 0.05 ml of each dilution were spotted on TSA and were incubated 4 days at 30°C.

[b] Inhibitory activity of the effector culture was determined by the agar overlay technique as described in Materials and Methods. Results are the averages of two experiments.

Table 2. Inhibitory activity of P. acidilactici on TSA as a function of incubation time and temperature

Incubation time, days[a]	Width of inhibitory zones, mm[b]		
	25°C	30°C	37°C
1	1	1	1
2	12	12	9
3	10	9	15
4	10	14	17
5	7	17	20
6	1	17	19

[a] Log-phase P. acidilactici cells in TSB were harvested, washed once in 0.1 M potassium phosphate (pH 7.0) and resuspended in one volume of fresh broth. A series of TSA plates was spot inoculated with 0.05 ml cell suspension and incubated at the designated temperatures.

[b] Inhibitory activities of the effector cultures on days 1 through 6 were determined by the agar overlay technique as described in Materials and Methods. Results are the averages of two experiments.

polyurethane stoppers during autoclaving. This phenomenon has been reported in an earlier study (4) and the toxic materials were isolated and characterized as volatile fatty amines.

Sterilization methods tested included saturation of the active broth with chloroform and subsequent aeration for solvent removal, filtration through a membrane filter (Nalgene Filter Apparatus, pore size 0.22 um), and heat treatment. Activity was absent in those samples treated with chloroform and in those which were filter sterilized, but no loss of activity occurred when the test tubes with broth were allowed to stand in a boiling water bath for 5 - 6 min (assayed by standard procedure). This treatment effected the killing of all producer cells, and was subsequently used as the method of sterilization prior to each bioassay.

Bacteriocin production was studied as a function of producer culture optical density at 600 nm ($O.D._{600}$) in TSB supplemented with glucose to concentrations of 0.25%, 0.50%, 1.0% and 2.0%. Log-phase cells grown in TSB were harvested by centrifugation, washed once in saline (0°C) and suspended in TSB (glucose omitted) to a final $O.D._{600}$ of 0.1. This suspension was used as the inoculum (10%) for each of the four media described above, and the cultures were incubated at 30°C. Samples were withdrawn at time intervals ranging between 6 and 12 h through 48 h and assayed according to standard procedure (Materials and Methods). The results are presented in Fig. 2. Bacteriocin production appears to commence either immediately or shortly after inoculation of the pediococci into fresh media, and is directly related to the cell density up to an approximate $O.D._{600}$ of 2.0. Further increases in optical density resulted in no or slight increases in bacteriocin titers. The maximum

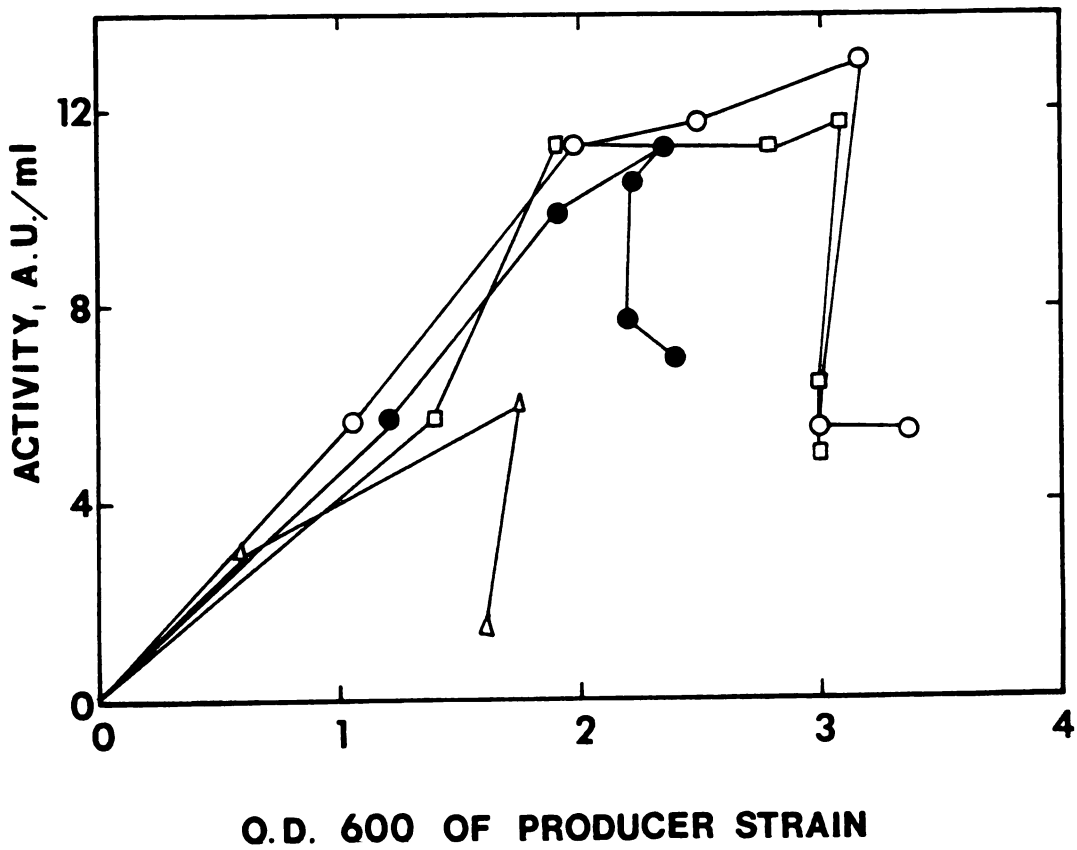

Figure 2. Inhibitory activity of P. acidilactici cultures as a function of producer cell density in TSB containing glucose at concentrations of 0.25 (Δ), 0.5 (•), 1.0 (□), and 2.0% (o).

cell density attained was dependent upon glucose levels in the media up to 1%, but where glucose was not limiting, bacteriocin titer was independent of glucose concentration.

Fig. 2 also shows rapid decreases in bacteriocin titers which occur as the cultures enter the stationary phases of their growth cycles. The change in bacteriocin titers with incubation time observed in the same experiment are depicted in Fig. 3. The initial loss of activity in the cultures, which may be as high as 50% within a 6 h interval in some instances, is followed by a leveling off period where the titers remain relatively constant throughout the remainder of the experiment. Such a dramatic decrease in bacteriocin levels might be due to the release of proteases into the medium, but efforts to demonstrate such activity in the culture supernatant by a method described by Costilow and Coulter (12) were unsuccessful.

The fact that the bacteriocin titer levels off before maximum cell density is attained suggests the possibility of a nutrient other than glucose being a limiting factor in bacteriocin production. However, supplementation of the medium with yeast extract at concentrations of 0.1%, 0.25%, or 1.0% resulted in no significant increase in titer. No inhibitory activity was produced when the Pediococcus culture was grown in LBS broth (BBL), a complex medium used for isolation and cultivation of lactobacilli (71), even though cell numbers were frequently greater than those reached in TSB. Growth of the producer culture under a variety of conditions including incubation at 37°C and 26°C, incubation at 30°C on a rotary shaker, and incubation at 37°C in an anaerobic chamber had little or no effect on the production of bacteriocin in broth media.

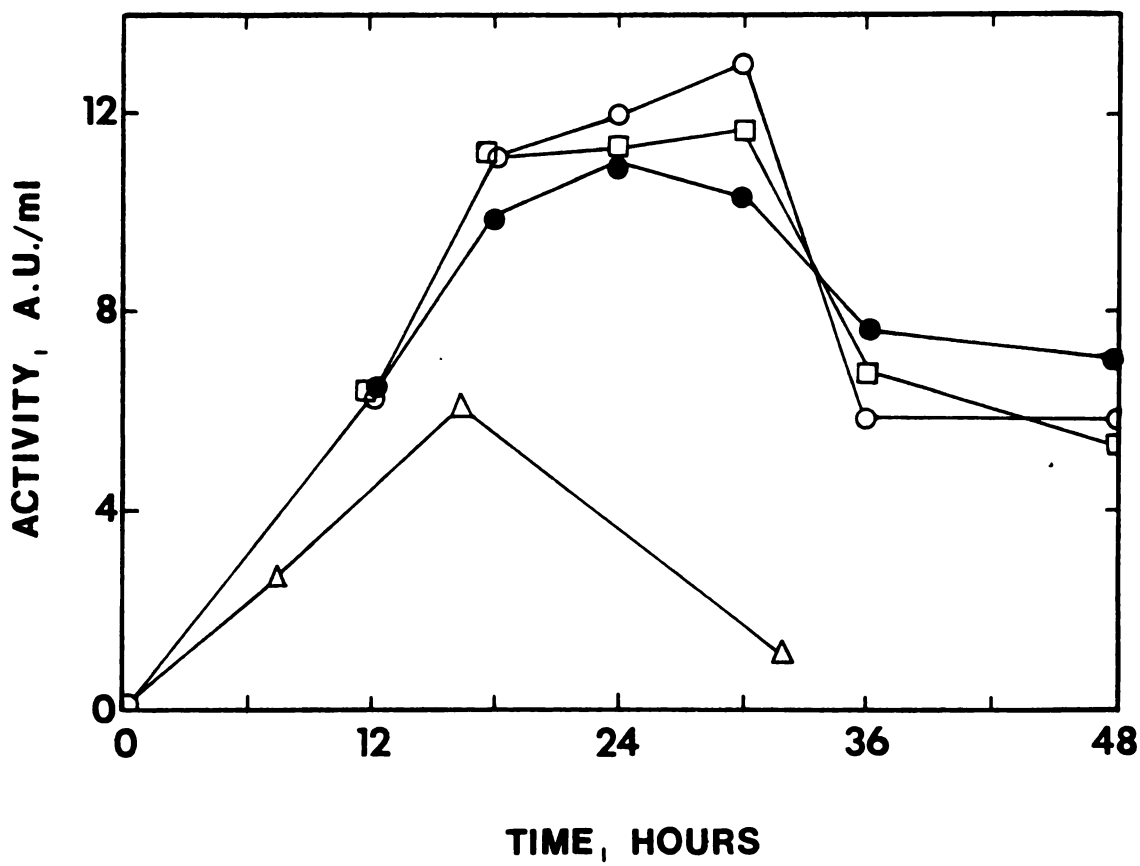

Figure 3. Inhibitory activity of P. acidilactici cultures in TSB containing glucose at 0.25 (Δ), 0.5 (•), 1.0 (□) and 2.0% (o) as a function of incubation time.

Cell-Free Preparations

Cell-free preparations of bacteriocins are necessary for meaningful studies of their production, their physical and chemical characteristics, and for operations leading to their concentration and purification. The procedure used most often during this study to obtain the activity in a cell-free state was a simple heat treatment of broth culture supernatant. Cultures of P. acidilactici were grown in TSB to late exponential-phase in flasks incubated statically at 30°C, and were centrifuged at 10^3 x g for 10 min to remove most of the cells. The resulting supernatant was sterilized in 5 - ml aliquots (18 x 125 mm test tubes) by submerging the tubes in a boiling water bath for 5 - 6 min. The preparations made by this procedure were then assayed according to standard procedure, and commonly had titers of 6 to 8 Arbitrary Units per ml (A.U./ml).

A second method by which cell-free preparations of bacteriocin was obtained was a freeze-thaw extraction of solid media (80). TSA plates were spread with 0.1 ml samples of a 100-fold dilution of log-phase producer culture and incubated 5 days at 30°C. The agar medium was collected and transferred to small Erlenmeyer flasks, which were then nearly submerged in dry ice-acetone baths for 10 min, or until the media had frozen. The flasks were removed from the baths and inverted at room temperature. The extracts produced as the media thawed were collected in test tubes and assayed according to standard procedure (Materials and Methods). The titers varied between 6 and 8 A.U./ml. In contrast, all attempts to demonstrate inhibitory activity in extracts of solid media which had supported growth of P. acidilactici without first

freezing the agar met with failure.

A number of methods were attempted to obtain bacteriocin activity either from producer cells or cell extracts. Log-phase cells grown in TSB were harvested, washed once in saline and eluted with 6M urea, 2M NaCl, or with solutions of triton X-100 (0.1%, 0.5% and 1.0%) in 2M NaCl containing 1 mM ethylene diamine-tetra-acetate. No inhibitory activity against L. plantarum was observed in cell extracts following disruption by sonication (MSE Sonicator), by a French pressure cell, or by a tissue homogenizer using the method of Dajani et al (15) for the isolation of viridins from alpha-hemolytic streptococci.

Physical and Chemical Characteristics

Stability in broth as a function of temperature: Loss of bacteriocin activity in TSB was a temperature-dependent phenomenon (Fig. 4). Thus, little decrease in titer occurred when active broth (sterilized by heat) was allowed to stand over a period of 10 days at 4°C, but a significant loss of activity occurred over the same period of time at 30°C. Inactivation of the bacteriocin at 37°C was only slightly more rapid than at 30°C.

In view of these data, it is interesting that the bacteriocin is resistant to inactivation by heat treatment at 100°C (Fig. 5). In this experiment, 10 mls of supernatant broth was transferred to each of 6 test tubes and placed in a boiling water bath for varying time intervals. Each tube was then allowed to cool to room temperature, and the broths were assayed according to standard procedure. There was only a slight decrease in titer in the broth that had received a 30 minute heat

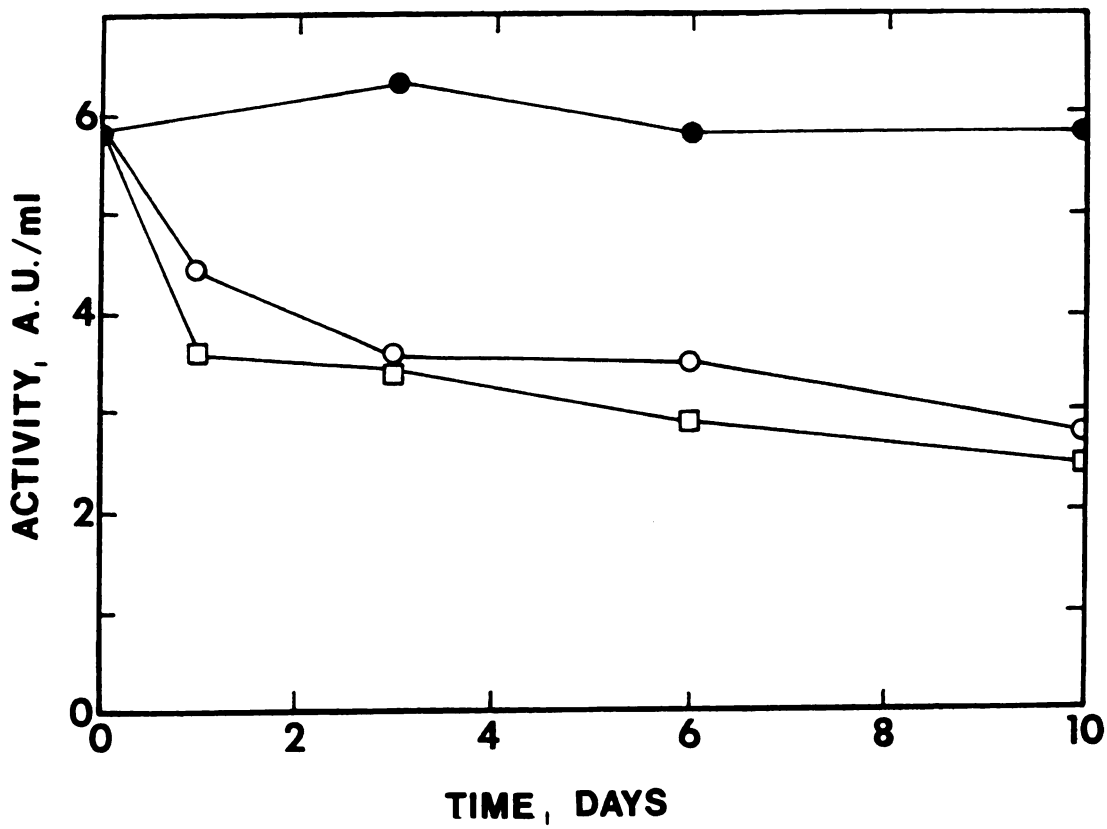

Figure 4. Loss of inhibitory activity of P. acidilactici culture supernatant with time at incubation temperatures of 25°C (•), 30°C (o), and 37°C (□).

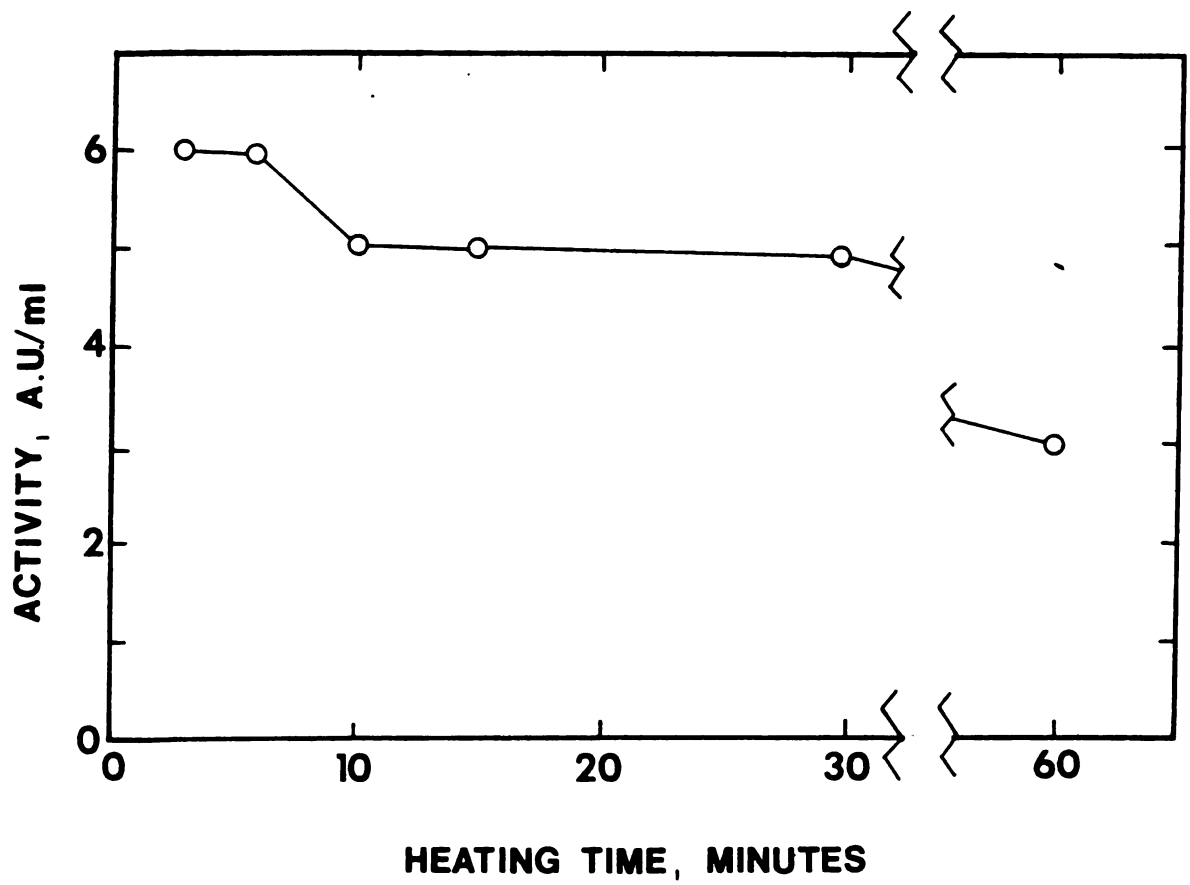

Figure 5. Stability of P. acidilactici inhibitory activity in TSB to heat treatment at 100°C.

treatment, and more than 50% of the original titer remained after exposure to 100°C temperatures for 60 min.

The bacteriocin was also stable to freezing. Active broth was either transferred to a freezer at -20°C and allowed to freeze slowly, or quick-frozen in a dry ice-acetone bath. The broth was later thawed and assayed for inhibitory activity by standard procedures. In both instances, the titer was equal to or greater than that of the untreated broth.

Dialysis: Samples (10 ml) of active Pediococcus culture supernatant broth were transferred to dialysis bags and were dialyzed overnight at 4°C against 1 liter of water; buffer (0.5% NaCl, 50 mM potassium phosphate, pH 7.0); and trypticase soy broth. One sample of broth was held overnight in a test tube at 4°C as a control. Each sample was assayed by standard procedure, and the results are presented in Table 3. Titers were significantly lowered in the 3 samples that were dialyzed, but not in the sample left as a control. However, the activity was not diluted 100-fold as might be expected if the bacteriocin was freely dialyzable. In a separate experiment, 10 mls of active broth was dialyzed overnight against 10 mls of fresh TSB at 4°C in a test tube. Although the activity was again reduced by 50%, the fresh broth surrounding the dialysis bag remained inactive, and activity was not restored to the dialyzed broth by combining the two fractions. These data suggest the bacteriocin is non-dialyzable across a semi-permeable membrane, but may in fact adhere to the dialysis tubing itself.

Chemical characteristics: The bacteriocin activity in broth medium is destroyed by treatment with at least two organic solvents. Loss of activity in broth and on plates following exposure to chloroform has

Table 3. Effect of dialysis on inhibitory activity in broth

Dialyzing medium	Titer, A.U./ml
None (control)	14.4
Distilled water	4.8
50 mM KPO_4 (pH 7.0)	5.2
TSB	4.4

already been mentioned. In addition, attempts were made to extract the active component from culture supernatant broth with ether. Broth and solvent were mixed (1:1) and allowed to stand for the separation of the two phases. The broth was then aerated for 30 min to achieve evaporation of any remaining solvent. An aliquot of fresh TSB was treated in an identical manner as a control. All samples were then assayed according to standard procedure. No inhibitory activity remained in the solvent-treated samples.

The bacteriocin was tested for its susceptibility to degradation by a variety of enzymes using a method similar to that described by Brubaker and Surgalla (10). TSA plates were inoculated with a single streak of log-phase P. acidilactici culture and incubated at 30°C for 5 days. The plates were then sterilized with chloroform vapors and overlayed with 5 mls of soft agar medium (TSA, 0.5% agar) freshly inoculated with L. plantarum (10^5 cells/ml). Small filter paper strips (0.5 x 2.0 cm) were soaked with 1% solutions of the enzymes to be tested (10 mM potassium phasphate, pH 7.2) and gently placed on the overlays perpendicular to the streak of the producer culture. The plates were then incubated overnight at 30°C as the enzymes diffused from the paper strips into the soft agar. An enzyme with the capacity to degrade the bacteriocin would protect the indicator cells near the paper strips, where the inhibitory zones would be significantly reduced relative to the untreated control plates. By this method, the bacteriocin was shown to be sensitive to the proteolytic action of pronase, but stable in the presence of trypsin, chymotrypsin, RNase, DNase and lipase.

The stability of the bacteriocin to pH extremes was studied by adjusting 10 ml aliquots of active broth to the desired pH with

concentrated HCl or NaOH. After 30 minutes of incubation at room temperature, each tube was brought back to neutrality and assayed for activity using the standard bioassay. However, results of these experiments were inconclusive, as control tubes of TSB treated in an identical manner were also antagonistic to growth of the indicator strain (presumably from the high salt content). Further studies on pH sensitivity may have to await the development of a procedure which would allow the removal of the salt without interferring with the expression of inhibitory activity by the bacteriocin.

Concentration

The bacteriocin titer of culture supernatant fluid was approximately doubled by the use of a dialysis culture. Effector cells were sealed within a dialysis bag containing 5 mls of TSB, which was then immersed in a liter of the same medium and incubated at 30°C. The titer of the culture supernatant fluid prepared in this way was 16 A.U./ml.

Many attempts were made to concentrate the activity in broth using techniques which are generally applied in the concentration and/or purification of proteins including ammonium sulfate precipitation, dehydration across a semi-permeable membrane, ultrafiltration, lyophilization, and ion-exchange chromatography with DEAE-cellulose. In each of these instances, the bacteriocin activity was either destroyed or reduced in the sample undergoing treatment. In addition to these methods, samples of broth medium containing bacteriocin was concentrated up to 20X in a rotary evaporator at 60°C. Interestingly, while the bacteriocin was not destroyed by this treatment, the titer of the concentrated broth

was only equal to that of the original untreated broth.

DISCUSSION

Preliminary work and a number of experiments described in this study have demonstrated that the properties of the inhibitory agent of P. acidilactici are consistent with many criteria generally attributed to the bacteriocins. The bactericidal nature of the activity is evident by the dramatic reduction in viable counts experienced by the indicator culture upon exposure to the bacteriocin in broth medium. Bacteriophage infectivity testing, performed with agar cut from inhibitory zones (85), demonstrated that the inhibitor is not a self-replicating, infective entity. The bacteriocin does not dialyze across membrane, and contains a proteinaceous moiety which is essential for biological activity.

The pediococcal inhibitor shared many characteristics with other bacteriocins of gram-positive organisms which tend to differentiate these molecules from the bacteriocins of gram-negative organisms. Relatively low titers of activity were detected throughout the study, and production could not be induced by treatment with ultraviolet radiation or with mitomycin-C. The bacteriocin is extremely resistant to inactivation by heat. In addition, an earlier study (26) has established a wide spectrum of activity for this bacteriocin. This is restricted to gram-positive bacteria. Such a spectrum is common among gram-positive bacteriocinogenic cultures (69).

It is not uncommon to experience difficulties in isolating and

purifying bacteriocins of gram-positive bacteria from liquid cultures (15, 29, 49, 53, 74, 84). Some bacteriocins require solid media for production, and many investigators have recovered the activity in solution by freeze-thaw elution from the agar (78). This technique was used successfully in this study, but the titer of the resulting solution was no higher than that of broth culture supernatant fluids.

As the titer of the culture supernatants were consistently low, a number of procedures were applied in an attempt to concentrate and/or purify the agent in solution. Most of these procedures (see Results) are those commonly employed in the concentration and purification of proteins. The activity was consistently lost or recovered at approximately the same titer as the starting material. These results might be explained by the possible existence of two or more distinct physical forms for the molecule, one of which may be inactive. This phenomenon is fairly common among bacteriocins of gram-positive bacteria (11, 22, 29, 35, 49, 75, 77, 83). The equilibrium between the monomers and the larger aggregates may be influenced by pH and ionic strength of the preparation (75, 77). Thus, if in the course of a concentration step the proposed equilibrium is shifted toward the inactive form, the demonstrable titer of the concentrate might remain at basal levels.

It is possible that a bacteriocin inactivator is synthesized concomitantly with the bacteriocin which may be responsible for the low titers expressed in broth media. Such inactivators have been found by several investigators (78), and are sometimes very effective in holding the activity of a given bacteriocin to a minimum. Thus, a protease may be released into the growth medium which degrades the pediococcal inhibitory agent. While no protease activity was demonstrated in the

culture supernatant fluids, bacteriocin inactivation did occur more rapidly in the presence of the producer cells than if the cells were removed by centrifugation and heat treatment (Figures 3, 4). These results might be expected if the protease itself was quickly inactivated after release, and would therefore be effective only if it was constantly synthesized and released by the Pediococcus culture. Alternatively, the bacteriocin inactivation may be due to the sudden excretion of an immunity protein by the producer cells into the medium at some time during their stationary phase of growth (corresponding to the reduction in titer). Removal of the cells prior to the release of such an inactivator would again greatly retard the loss of activity in the supernatant. Indeed, the failure to enhance the bacteriocin titer through protein purification techniques may be due to an accompanied concentration of an immunity protein present in the broth.

The unsuccessful attempts to concentrate the bacteriocin in broth media might also be explained if the agent was hydrophobic. It is feasible that such a molecule would only be soluble in water to a certain level. Upon concentration, the bacteriocin may precipitate out of solution in an inactive aggregate of some form. Clark et al (11) found two Clostridium bacteriocins which were soluble in concentrated aqueous ethanol. The investigators were able to purify the bacteriocins by a procedure which involved the binding of a nonionic detergent (Triton X-100) to the inhibitory proteins. The protein-detergent adducts were purified by gel filtration through Sephadex 6B, and the bacteriocins were then freed of Triton X-100 by chromatography on Sephadex LH-20. A similar procedure may offer a possible solution to the problems encountered in the concentration of the P. acidilactici bacteriocin.

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