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Bacteriocin production and susceptibility of acinetobacter calcoaceticus

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BACTERIOCIN PRODUCTION AND SUSCEPTIBILITY OF ACINETOBACTER CALCOACETICUS

by
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THESIS

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INTRODUCTION

Acinetobacter calcoaceticus[1] is a common bacterium in hospital-associated infections and thus is of epidemiological concern (2-6). Like *Pseudomonas aeruginosa*, and many of the Enterobacteriaceae, *Acinetobacter* is an opportunist. It normally does little harm to healthy individuals, but is capable of causing serious infections in compromised patients, such as those with impaired humoral or cellular immunity, as in cancer or Hodgkins's disease (7), those who have had their primary defense mechanisms weakened, as in the case of burns (8) or trauma, and those who have been subjected to manipulative medical procedures, such as surgery, catheterization, and respiratory therapy (9).

The primary habitat of *Acinetobacter* appears to be soil (10-12), water (10, 12, 13), and sewage (11), although it also has been isolated from frozen foods (14) and dairy products (15), and within hospitals from respiratory equipment (4), containers of quaternary ammonium disinfectants (16), unsterile cotton balls (16), and hyperalimentation fluids(17). *Acinetobacter* exists as a commensal in the normal human flora (2, 5). It has been found quite often

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[1] formerly known as *Herellea vaginicola*, *Mima polymorpha*, *Achromobacter anitratum*, *Achromobacter lwoffi*, *Bacterium anitratum*, and *Acinetobacter anitratum* (1).

on the skin and occasionally in the upper respiratory tract (18). It frequently is isolated from numerous types of routine clinical specimens, including skin (5, 19), wounds (8, 20), mucous membranes (5), urine (21), feces (2), blood (22), and cerebrospinal fluid (23, 24). Quite often it is difficult to determine whether isolation of Acinetobacter from a patient indicates contamination or infection since the overall pathogenic role of Acinetobacter has not been established.

The nutritional requirements of Acinetobacter are not complex (12), and it can survive in both wet and dry environments (2). For these reasons, there may be numerous reservoirs of colonization or infection by this organism within a hospital, including hospital personnel, other patients, and environmental factors, as well as the patient's own body flora. With this range of possibilities, numerous strains of Acinetobacter may be isolated from clinical specimens. For epidemiological purposes, it is important to establish the relationships, if any, that exist between these isolates and, if possible, identify a common mode of transmission to the patients from a central source of contamination.

There are several ways to differentiate bacterial strains within a species. These methods include (25, 26); 1) the evaluation of gross phenotypic characteristics, 2) antibiotic susceptibility testing, 3) serological typing, 4) bacteriophage susceptibility, and 5) bacteriocin susceptibility and production. Although testing for gross pheno-

typic characteristics and antibiotic susceptibility is fast and relatively simple, these characteristics are not highly stable (27). In addition, these tests are not always discriminatory in distinguishing different strains of bacteria within a species. Serological typing of Acinetobacter has been somewhat successful (28, 29); however, the lack of commercially available antisera, combined with the variety of different antigenic types, make this too complicated and expensive a procedure for routine clinical laboratory use. A few Acinetobacter phages have been isolated (30-32), but a bacteriophage typing system has not been documented. In 1960, Pickett (33, 34) described the occurrence of what may have been bacteriocin activity of Acinetobacter, but no further attempts to evaluate this phenomenon have been reported.

Bacteriocin production is believed to be a relatively stable characteristic (26) and testing for bacteriocin activity often can differentiate otherwise identical strains. For these reasons, and because a bacteriocin typing system is relatively simple to maintain once it is established, such a system has advantages over other typing methods. Bacteriocin typing systems have been developed for several bacterial species, including Pseudomonas aeruginosa (25, 26, 36 - 43), E. coli (44), Shigella sonnei (45, 46), Neisseria meningitidis (47), and Serratia marcescens (48 - 50).

Bacteriocins are antibiotic-like substances of protein nature produced by one bacterial strain, and are active against other strains of the same or closely related species. They were first observed by Gratia in 1925 (51), and later by Fredericq (52, 53), both working with coliforms. Since these initial observations, numerous species of both gram positive and gram negative bacteria have been shown to produce bacteriocins, only a few of which have been studied extensively. The fundamental mechanism of bacteriocin activity involves the adsorption of a bacteriocin molecule to a specific receptor on the surface of a susceptible cell. Adsorption is followed by a chain of events which alter the host cell's metabolism, ultimately resulting in the destruction of the cell (54).

The protein nature of bacteriocin molecules and their requirement for specific receptors on the surface of susceptible bacterial cells constitute the main differences between bacteriocins and classical antibiotics (54, 55). Bacteriocin production and susceptibility, and bacteriophage development and susceptibility are similar phenomena. The obvious difference between bacteriocins and bacteriophages is the ability of bacteriophages to multiply following infection of susceptible cells.

There are several methods that may be used to detect bacteriocin activity (56). These include growing producers on agar solidified media or broth media with and without the use of inducing agents. The environmental conditions most

suitable for bacteriocin production and susceptibility differ for various organisms, and must be determined by trial and error (57). Distinguishing the conditions most suitable to the individual organism is the most important step in developing a bacteriocin typing system. Once a satisfactory method is found, the evaluation of a broad sampling of strains within that species would make it possible to select a representative number of producers and/or indicators that could be used in the development of a typing schema. In this way, clinical isolates could be typed by bacteriocin production against known indicators or by bacteriocin susceptibility against known producers. Because the indicators lack stability, typing by bacteriocin production has been the preferred system for other bacteria (49, 56).

Although bacteriocin activity is usually confined to strains within a species, what was believed to be bacteriocin activity has been shown to cross species and, in some cases, even genus barriers. Usually, this activity is greatest against those organisms that are phylogenetically most closely related to the bacteriocin-producing organism (58 - 61).

Both Pseudomonas aeruginosa and Acinetobacter are included among the glucose non-fermenting gram negative bacilli, and are thought to be closely related on the phylogenetic scale (62, 63). Approximately 90% of Pseudomonas

aeruginosa strains are capable of producing bacteriocins (specifically termed pyocins), and there is considerable diversity in activity among the pyocins produced (26, 37, 58, 64). Certain genera and species of organisms, other than Pseudomonas aeruginosa, have been shown to be susceptible to pyocins (61, 65). Even though Acinetobacter has not been documented as one of these organisms, it is possible that it too, may be susceptible.

The purpose of this study was to investigate bacteriocin production and susceptibility of Acinetobacter. The feasibility of utilizing this method of "fingerprinting" Acinetobacter isolates was also evaluated. Seventy strains of Acinetobacter, most of which were clinical isolates, were tested. Both agar and broth methods of bacteriocin production were evaluated under a variety of environmental conditions.

The susceptibility of these Acinetobacter strains to pyocins produced by 20 strains of Pseudomonas aeruginosa was also tested using both agar and broth methods. The activity of pyocins against Acinetobacter and Pseudomonas aeruginosa was compared with respect to their physical and chemical characteristics and kinetics of killing.

MATERIALS AND METHODS

Bacteria

Seventy strains of *Acinetobacter calcoaceticus* were used in this study.

Seventeen strains of *Acinetobacter calcoaceticus* var. *anitratum*, all of which were different serotypes (28), were obtained from the Center for Disease Control. These included serotypic strains 2F, 3F, 4F, 5F, 7F, 8F, 9F, 10F, 5J, 8J, 12J, 13J, 15J, 17J, 18J, 19J, and 20J. In this study these were designated strains 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 15, 16, 17, 18, and 19, respectively.

Forty-nine isolates of *Acinetobacter calcoaceticus* (both *lwoffi* and *anitratum* varieties), designated strains 20 to 35 and 41 to 73, 26 isolates of *Pseudomonas aeruginosa*, designated strains 101 to 126, and three isolates of *Pseudomonas maltophilia*, designated strains 127 to 129, were obtained from various clinical specimens processed in several clinical microbiology laboratories in the San Francisco Bay Area.

Four strains of *Acinetobacter calcoaceticus* (3 *anitratum* and 1 *lwoffi* varieties), designated strains 37 to 40 were provided by the Bacteriology Section of the California State Department of Health.

Acinetobacter auxotroph trpE27 was used in the transformation assay (66, 67).

Media

Dehydrated nutrient agar (NA), trypticase soy broth (TSB), trypticase soy agar (TSA), brain heart infusion (BHI) broth, brain heart infusion (BHI) agar, MacConkey agar (Mac), and triple sugar iron agar (TSI) were obtained from BBL. Dehydrated heart infusion (HI) agar, Mueller-Hinton broth (MHB), Mueller-Hinton agar (MHA), nitrate broth, and motility GI media were obtained from Difco. All these media were prepared according to the manufacturer's specifications.

Dehydrated agar was obtained from Difco.

Ten percent lactose slants were prepared by adding 50% lactose (Difco) to phenol red broth base (BBL), agar, and water.

Oxidative-fermentative (OF) glucose media, which contained a final concentration of 1% glucose was made using Difco OF media and adding glucose (Difco) to it in the form of a 10% solution.

One percent glucose broth was prepared using the formulation for Andrade's carbohydrate broths as described in the Manual of Clinical Microbiology (1).

Six to seven percent sheep blood agar plates (BAP) were prepared using TSA base (BBL) and supplementing with yeast extract (Difco) to give a final concentration of 1% yeast extract.

Preparation and Storage of Stock Cultures (68)

The organisms to be stored were grown on nutrient agar slants overnight at 35 C. Approximately 1 ml of sterile defibrinated sheep blood was pipetted onto each slant and the growth was scraped off with the pipette and mixed with the blood. The blood-organism suspensions were pipetted into sterile one dram vials that were half full with alundum. The vials were shaken to uniformly coat the alundum particles. Prior to storage at -50 C to -70C, the samples were quick-frozen by placing them in a dry ice-acetone bath for approximately 5 minutes. To retrieve stock organisms, a few grains of the coated alundum particles were dislodged, transferred to BHI broth and incubated.

Working cultures were maintained at 4 C on nutrient agar slants. These were transferred every 4 to 6 weeks.

Identification of Organisms

Transformation Assay The transformation assay described by Juni (65, 69) was used to confirm the identification of the Acinetobacter strains used in this study.

The crude DNA used in this assay was prepared by inoculating a small amount of bacterial cell paste, taken from overnight growth on a HI agar plate, into 0.5 ml of 0.05% sodium dodecyl sulfate in standard saline citrate solution (0.15M sodium chloride, 0.015M sodium citrate) contained in a 13 by 100 mm screw-capped tube. Care was taken to avoid

applying cell paste to the side of the tube where it might not be mixed into the detergent. The bacteria-detergent suspension was vortexed and then heated in a 60 C water bath for 15 to 60 minutes which allowed the cells to lyse and release DNA. These sterile DNA preparations could be stored indefinitely in the refrigerator provided the caps were screwed on tightly to prevent evaporation.

The transformation assay involved marking off an HI agar plate into 9 sectors. A small amount of cell paste of Acinetobacter auxotroph trpE27 grown overnight on a HI agar plate at 35 C was placed in the center of a sector. A sterile loopful (approximately 2 mm in diameter) of crude DNA to be tested was used to suspend the cells previously placed on the plate, and the DNA-cell mixture was spread in a circular area approximately 10 mm in diameter. A second loopful of DNA was spread over another sector to serve as a sterility control. The auxotroph was inoculated on one sector per plate to test its stability. Four DNA samples were tested per plate, each accompanied by a DNA sterility control respectively. Following 2 to 5 hours incubation at 35 C (by which time some growth was visible), a generous portion of each growth area was streaked onto a lactic acid mineral agar plate (eight sectors per plate). One sector of each plate was streaked with auxotroph trpE27 non-DNA-treated control cells, which should not grow on this minimal media. This streaked plate was incubated for 15 to 48 hours

at 35 C. After incubation, the streaked areas were observed for colonies derived from cells of trpE27 that were transformed to prototrophy during growth in the presence of DNA on the HI agar plate.

According to Juni, the ability of the test organism to transform Acinetobacter auxotroph trpE27 to prototrophy indicated that it was a strain of Acinetobacter. Likewise, failure to do so indicated that it was not.

Biochemical Tests The Acinetobacter strains used in this study were identified biochemically by the method described by Samuels, et. al. (70) and according to the characteristics listed in Bergey's Manual (70).

Specifically, strains were tested for: oxidase production; nitrate reduction; motility; growth on nutrient agar; growth on MacConkey's agar; hemolysis of 6% sheep red blood cells contained in blood agar plates; oxidative utilization of, and failure to ferment, glucose, lactose and sucrose as observed on TSI, 10% lactose, 1% glucose, and OF glucose.

The identity of Pseudomonas aeruginosa strains was confirmed by their ability to produce oxidase and pyocanin, and by their ability to utilize glucose, lactose, and sucrose oxidatively but not fermentatively.

Susceptibility Testing

The antimicrobial susceptibility of the Acinetobacter strains was tested and defined according to the method described by Bauer, et. al. (72).

Serological Testing with Pseudomonas aeruginosa Antisera (73)

A set of antisera prepared against the somatic antigens of 17 tentative serotypes of Pseudomonas aeruginosa (Difco) was used to serotype some of the Acinetobacter and Pseudomonas aeruginosa isolates used in this study.

The procedures followed were those recommended by Difco for these particular antisera.

The lyophillized antisera were reconstituted with 1 ml distilled water. Working solutions were prepared weekly by making a 1:10 dilution of the rehydrated antisera in 0.85% saline containing 1:10,000 merthiolate. All antisera were stored at 2 - 8 C.

The organisms to be tested were grown on BAP overnight at 35 C.

In testing unheated cells as antigens, one drop of a heavy suspension of organisms in saline was added to one drop of diluted antisera on a glass plate. The plate was rotated by hand for one minute and examined for agglutination.

In testing heated cells as antigens, the BAP were streaked in such a manner as to obtain confluent growth. As much of this growth as possible was washed from the plates with 10 ml of 0.85% saline and autoclaved at 121 C for 30 minutes. Following centrifugation at approximately 3000 x g for 5 to 10 minutes, the supernatant was discarded and the bacteria were resuspended in 0.75 ml merthiolate saline

(0.85% saline, 1:10,000 merthiolate). These stable heated antigens were agglutinated as described above.

Bacteriocin Production and Susceptibility

In this investigation, bacteriocin production on both agar-solidified media and broth media were evaluated. The procedures followed were essentially those that had been successfully developed for Pseudomonas aeruginosa (26, 37, 41) and Serratia marcescens (49, 50).

Agar Media These methods involve growth of selected producer strains on agar surfaces, chloroforming the growth to kill viable cells, and either cross-streaking with indicator organisms or overlaying with agar seeded with indicator organisms. The main variables in agar methods include the type of media used, the length and temperature of incubation, and the method of application of both the producer and the indicator organisms.

Bacteriocin activity is observed as areas of clearing within the indicator growth.

In this study, to rule out clearing due to phage rather than to bacteriocin, the zones of inhibition (particularly the edges) were examined for the absence of plaques. In addition, some strains were checked by transferring a portion of the cleared agar to another indicator lawn to observe for transfer of lysis.

Scrape-and-Streak Method The procedure of Darrell and Wahba (36) was used with modifications as indicated below.

Nutrient agar prepared in glass petri dishes was inoculated with the potential bacteriocin producer by applying 3 evenly spaced transverse streaks approximately 1 cm wide. Following overnight incubation at 35 C, the plates were exposed to chloroform vapours for 1 hour. This was accomplished by placing filter paper soaked with chloroform into the lid and inverting the bottom of the dish over this. The growth was then scraped off with a tongue blade, and the plates were re-exposed to chloroform vapours for another hour. The filter paper was removed, and the plates were left with lids ajar for one hour to allow the residual chloroform to evaporate.

A loopful of each indicator organism, grown in 10 ml BHI broth overnight at 35 C, was streaked across the plates at right angles to the lines of the original producer inoculum. As many as 13 different indicators could be applied per plate. Following another 18 to 24 hours of incubation at 35 C, the plates were examined for evidence of inhibition of growth of the indicator strains.

Modifications of the above procedure involved: 1) using HI agar instead of nutrient agar; 2) growing the indicator organisms in TSB instead of BHI.

Agar Overlay Method The method of Graham (74) was used.

The surface of dry nutrient agar in glass petri dishes was inoculated with the cell paste of potential bacteriocin producers by means of a replicator which applied spots

(approximately 1.5 cm in diameter) of organisms to be tested. As many as 27 producers were tested per plate. Following overnight incubation at 35 C, the plates were exposed to chloroform vapours for one hour as described previously; however, the growth was not scraped off the plate. The plates were then left with their lids ajar for an additional 30 to 60 minutes to allow the residual chloroform to evaporate. Indicator organisms were grown overnight in 5 ml TSB at 35 C. Then 0.1 ml of each suspension was added to 5 ml of melted, soft agar that had been cooled to approximately 50 C. This was mixed, poured over the chloroformed bacteriocin plate, allowed to harden, and incubated overnight at 35 C.

The above procedure was modified to standardize the inoculum of indicator organisms by adjusting the overnight broth suspension to an optical density (O.D.) of 0.05 at 550 nm prior to seeding the soft agar.

The soft agar was prepared by adding 10 gm beef extract (Difco), 10 gm Bacto peptone (Difco), 5.0 gm sodium chloride and 4 gm agar to one liter of distilled water.

Broth Media These methods involve growth of the potential producer strains in a suitable broth, removal of viable organisms by chloroforming or filtration, and dropping these bacteriocin preparations onto indicator lawns. There are several variables in broth methods of bacteriocin production. The determination of the most suitable broth

for bacteriocin production by particular bacteria is of prime importance. Other significant considerations include: the use of inducing agents, such as x-rays or mitomycin C; the length and temperature of incubation; the method of sterilization of the bacteriocin solutions. In preparation of the indicator lawn, the choice of media is again an important factor as is the temperature and length of incubation. Another variable involves the method of inoculating the indicator organisms onto the agar surfaces.

In these experiments, the method used for applying the bacteriocin preparations onto the indicator lawns involved dropping them from sterile Pasteur pipettes, each drop delivering approximately 0.04 ml. An uninoculated tube of broth was carried through each step of the bacteriocin preparation as a control and dropped onto each test plate. It was essential that the indicator lawns had dried prior to application of the bacteriocins. After the bacteriocin drops had dried, the plates were incubated. The next day they were examined for bacteriocin activity as evidenced by areas of inhibition in the indicator lawn that were greater than that of the control. The degree of inhibition was judged as follows: 4^+ = complete clearing; 3^+ = 1 to 10 colonies within the zone of inhibition; 2^+ = 10 to 50 colonies; 1^+ = 50 to 100 colonies; H = questionable inhibition, barely greater than that of the control; 0 = no inhibition apparent. The control was usually discernible

by a mark where the drop had been applied to the indicator lawn. Those zones reported as H showed slightly greater inhibition.

Again, to make certain that the zones of clearing were due to bacteriocin and not to phage, the zones were examined for the absence of plaques. In some cases, 10-fold dilutions of the bacteriocin preparations were made and dropped onto indicator lawns. Bacteriocin activity gradually diluted out whereas phage activity would not have done so as clearly. Also, plaques would have become evident as phage was diluted (75).

Comparisons of activity of different bacteriocin preparations were carried out on the same day, as there was considerable loss of activity from day to day. When necessary, bacteriocin preparations were stored at 4 C.

Broth with Mitomycin Induction The method of Farmer (49, 50) was used with modifications as indicated below.

The organisms to be tested for bacteriocin production were grown overnight on a TSA plate. A small amount of this growth was inoculated into 4 ml TSB and incubated in a 32 C water bath for 24 hours. Then 0.7 ml was removed and added to 3.3 ml TSB. Following mixing, the tube was incubated for 1 hour in the 32 C water bath. Then 1 ml of a 25 ug/ml filter-sterilized mitomycin C (Sigma Chemical Co.) solution in TSB was added. The tube was mixed and re-incubated for an additional 5 hours. It was during this

final incubation period that cells were lysed and bacteriocin released. The bacteriocin preparations were sterilized by adding 0.3 ml chloroform and mixing vigorously. The tubes were centrifuged at approximately 3000 x g for 10 minutes to remove cellular debris. The supernatant was pipetted into sterile glass tubes with the lids left ajar for at least one hour to allow residual chloroform to evaporate.

In preparation of the indicator lawns, organisms were grown in 4 ml TSB overnight at 35 C. An aliquot of this growth was placed into another tube containing 4 ml TSB to achieve a turbidity which approximated an O.D. of 0.10 at 550 nm. Then 0.1 ml of this suspension was added to 3 ml of 0.7% aqueous agar that had been melted and cooled to approximately 50 C. Following mixing, this was overlaid onto a plate of BHI agar and allowed to harden. The bacteriocin preparations were applied and the plates were incubated for 18 to 24 hours at 35 C prior to examination for inhibition.

Modifications in preparation of the bacteriocins included: 1) carrying out all incubations at 35 C; 2) varying the concentration of mitomycin C added from 0 to 25 ug/ml; 3) adding 1.5 ml of an overnight broth suspension to 2.5 ml TSB in the initial step, instead of 0.7 ml suspension to 3.3 ml TSB.

Modifications in preparation of the indicator lawn included: 1) growing the indicator organisms overnight at 32 C in 4 ml TSB; adding 5 drops of this to 0.5 ml TSB, incubating 2 to 4 hours, and mixing 0.001 ml (calibrated loop) of this with 3 ml aqueous agar; 2) picking 5 colonies of indicator organism grown on a BAP overnight into 0.5 ml TSB, incubating 4 hours at 35 C, adding an additional 0.5 ml TSB to the suspension, and removing 0.001 ml into 3 ml aqueous agar; 3) growing indicator organisms overnight in 5 ml TSB at 35 C, removing 0.01 ml (calibrated loop) into 4 ml TSB, flooding a TSA plate with this, and removing the excess broth prior to drying and dropping bacteriocin preparations.

Broth with Mitomycin Induction and Aeration The method described by Farmer (37) was used with modifications as below.

The organisms to be tested for bacteriocin production were grown on TSA agar plates for 18 to 24 hours at 35 C. Some of the cell paste was removed with a cotton swab and agitated with 10 ml TSB until the turbidity approximated an O.D. of 0.40 at 550 nm. This suspension was decanted into 20 ml of TSB in a 125 ml Erlenmeyer flask and grown with constant shaking in a 32 C water bath. After 2 hours, 1 ml of a sterile solution containing 30 ug/ml mitomycin C dissolved in TSB was added, and the shaking was continued

for another 5 hours. This solution was then transferred into a 50 ml glass tube containing 5 ml chloroform, shaken vigorously for 20 seconds and centrifuged for 15 to 20 minutes at approximately 5000 x g. The top layer was pipetted into sterile glass tubes with the lids left ajar for at least one hour (depending on the volume contained in each tube) to allow evaporation of residual chloroform.

Each indicator was grown overnight on a TSA plate at 35 C. A portion of this cell paste was agitated with TSB until the turbidity approximated an O.D. of 0.40 at 550 nm. A TSA plate was flooded with 2 ml of this suspension and the excess liquid was removed. Following application of the bacteriocin preparations onto these lawns, the plates were incubated for 18 to 24 hours at 35 C.

Modifications of the above procedure in preparing the bacteriocins included: 1) not adding any mitomycin C; 2) sterilization of the preparations by centrifugation to remove cellular debris and passing the supernatant through a 0.22 micron pore size cellulose acetate filter (Nalge Corp.).

Modifications in preparing the indicator lawns involved: growth of the indicator organisms overnight in 5 ml TSB at 35 C, removing 0.01 ml of this suspension into 4 ml TSB, pouring this suspension onto a TSA plate, and removing the excess liquid prior to drying and dropping of the bacteriocin preparations.

Broth Using Medium 81 The method of Jones, et. al. (26, 41) was used.

The potential bacteriocin producers were inoculated into 10 ml medium 81 (TSB without glucose plus 1% potassium nitrate) and grown at 32 C for 18 hours. One milliliter of chloroform was added to each tube. The tubes were shaken vigorously for 10 seconds, allowed to stand at room temperature for 10 minutes, then shaken again. Following centrifugation for 10 minutes at approximately 3000 x g, the supernatant was removed and pipetted into sterile glass tubes with the lids left ajar to allow residual chloroform to evaporate.

The indicators were inoculated into 4 ml of medium 81 and incubated for 24 hours at 32 C. A 0.01 ml aliquot was mixed with 4 ml medium 81 and poured onto a dry TSA plate. The excess liquid was pipetted off. The bacteriocin preparations were dropped onto the indicator lawns and the plates were incubated for 18 to 24 hours at 35 C.

The only modifications of the above procedure were in respect to preparation of the indicator lawns. These involved: 1) growing indicator organisms at 35 C rather than 32 C; 2) growing and diluting indicator organisms in TSB rather than in medium 81.

Assessment of Pyocin Activity

The pyocin preparations used in these studies were obtained by the mitomycin-induced aeration method of Farmer (37) as described above.

Determination of Titers of Activity (37) Ten-fold dilutions of pyocins were made in TSB and dropped onto the indicator lawns. The titer was defined as the reciprocal of the highest dilution demonstrating 2+ or greater activity. Units of activity were synonomous with the titer.

Thermal Stability (76) One milliliter aliquots of undiluted pyocin were placed in sterile glass tubes and incubated for 30 minutes at 50 C, 60 C, and 70 C, respectively, prior to dropping them onto indicator lawns.

Trypsin Sensitivity (77) A 1% (w/v) trypsin (Miles Chemical Co.) solution in 0.1 M phosphate buffer (pH 7.4) was adjusted to pH 7.4 and filter sterilized. Then 0.2 ml of this solution was added to 1 ml aliquots of undiluted pyocin or, in certain cases, to various dilutions of pyocin, and incubated in a 37 C water bath for 1.5 hours. The treated pyocin preparations were then dropped onto indicator lawns.

Chloroform Sensitivity Prior to sterilization, the pyocin preparations were divided into two portions. One portion was chloroform-sterilized as described above. The other portion was centrifuged to remove cellular debris, and the supernatant was passed through a 0.22 micron pore size

cellulose acetate filter. This sterile preparation was dropped onto indicator lawns, as was the chloroform-sterilized preparation, and their activity compared.

Kinetic Studies

The kinetics of pyocin killing of Acinetobacter as compared to Pseudomonas aeruginosa were examined (56). Ten-fold dilutions of an overnight growth in TSB of the organisms to be tested were prepared until a 10^{-7} dilution was obtained. Two 0.1 ml aliquots of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated onto 2 BAP respectively in order to determine the number of viable organisms contained in the overnight suspension and in each dilution.

A 0.9 ml sample of an appropriate dilution of test organism was placed in a 16 by 125 mm sterile plastic tube. At 00 seconds, 0.1 ml of undiluted pyocin was added and the tube was immediately vortexed. At various time intervals 0.1 ml samples were removed from the reaction vessel and immediately inoculated into 9.9 ml TSB in order to prevent further bacteriocin action. From these tubes, 0.1 ml aliquots were plated onto 2 BAP and incubated overnight at 35 C to obtain viable counts.

The pyocin-Acinetobacter reactions were carried out in various broths, including TSB, TSB-Ca^{++} (10 mg/100 ml anhydrous calcium chloride), Mueller-Hinton broth (MHB), MHB-Ca^{++} (10 mg/100 ml anhydrous calcium chloride), and 0.85% saline. The pyocin-Pseudomonas reactions were carried

out in TSB and saline. This was accomplished by diluting the bacteria to 10^{-3} in the respective broth and adding pyocin. The results of pyocin killing, as determined from viable counts at varying time intervals, were compared.

EXPERIMENTAL

I. Identification of Organisms

A. Transformation Assay

The ability of DNA extracted from the test organisms to transform the Acinetobacter auxotroph trpE27 to prototrophy, was used to confirm the identity of 77 isolates originally designated as Acinetobacter by the cooperating laboratories. Of these 77, 70 were able to transform. The 7 organisms which failed to do so were biochemically identified as belonging to genera other than Acinetobacter. This interspecies transformation established the genetic relatedness among the Acinetobacter strains studied.

2. Biochemical Tests

By the use of biochemical tests, 4 of the 70 Acinetobacter strains were variety lwoffi, and 66 were variety anitratum. The biochemical test results, expressed as numbers positive and percent positive are shown in Table 1.

The main differentiating characteristic between the two varieties of Acinetobacter is their utilization of carbohydrates with resulting acid production. Variety anitratum is able to form acid from several carbohydrate substrates whereas variety lwoffi is not (71).

Fifty-three of the 70 Acinetobacter strains in this study were clinical isolates from local hospitals. Of

these, only 4 were variety lwoffi. Previous clinical studies have shown that variety anitratum does appear more frequently than variety lwoffi (78), thus supporting our findings.

Table 1. Biochemical reactions of 70 strains of Acinetobacter calcoaceticus

Test	Sign	No.+	%+	var. anitratum No.+	var. anitratum %+	var. lwoffi No. +	var. lwoffi %+
TSI	alk/alk[a]	70	100	66	100	4	100
10% lactose	acid	65	92	65	98	0	0
1% glucose	acid	65	92	65	98	0	0
nitrate	-[b]	0	0	0	0	0	0
oxidase	-	0	0	0	0	0	0
motility	-	0	0	0	0	0	0
OF glucose	Ox[c]	66	94	66	100	0	0
NA growth	+[d]	70	100	66	100	4	100
Mac growth	+	60	86	58	89	2	50
Hemolysis	beta	2	3	2	3	0	0

[a]alkaline; [b]negative reaction; [c]oxidative utilization; [d]positive reaction

II. Susceptibility Studies

Antibiotic susceptibility of the 70 Acinetobacter strains was measured using the standard Kirby-Bauer disc diffusion method (72). No correlations between patterns of susceptibility to specific antibiotics and susceptibility to pyocins were noted.

All 70 Acinetobacter strains were resistant to cephalothin and susceptible to colistin. Most strains were susceptible to tetracycline, kanamycin, gentamicin, and tobramycin. The most typical pattern was resistance to ampicillin, cephalothin, chloramphenicol; susceptibility to tetracycline, kanamycin, colistin, gentamicin, and tobramycin; and either intermediate susceptibility or susceptibility to carbenicillin. Thirty-seven percent of the strains exhibited this pattern. There was considerable variation in the patterns demonstrated by the other 63 percent of the strains.

III. Cross-Reactivity of Acinetobacter Strains with Antisera to Pseudomonas aeruginosa

The 33 Acinetobacter strains that were susceptible to pyocins were tested for their ability to agglutinate in antisera against 17 diffferent serotypes of Pseudomonas aeruginosa. When unheated cells were used as antigen, no reactions were observed. When autoclaved cells were used as antigen, 7 of the 33 Acinetobacter strains tested showed weak agglutination in antiserum of serotype 9 and antiserum of serotype 14. These Acinetobacter strains were #5, 9, 19, 21, 28, 38, and 49.

The fact that several of the Acinetobacter strains agglutinated weakly in antisera prepared against Pseudomonas aeruginosa, suggests a possible antigenic relationship be-

tween these two genera. Two Pseudomonas aeruginosa serotypes, 9 and 14, were involved in all cases and agglutination was demonstrated only when autoclaved cells of Acinetobacter strains were used as antigens. The reason for the negative reactions with unheated antigens may be explained, as mentioned by the manufacturer (Difco), by the fact that antisera were prepared against somatic antigens and not whole cell antigens. It is possible that non-reacting, heat-labile antigens cover the cross-reacting somatic antigens.

It has been demonstrated previously that Pseudomonas aeruginosa possesses antigens with varying degrees of specificity (79). It was found that at least 6 antigens were widely distributed among strains of Pseudomonas and related genera, including Achromobacter.

IV. Evaluation of Bacteriocin Production and Susceptibility --Acinetobacter against Acinetobacter

A. Agar-solidified Media

1. Scrape-and-Streak Method

Potential producer strains of Acinetobacter were grown as wide transverse streaks on HI agar plates. Following exposure to chloroform vapours to kill viable cells, the growth was scraped off and the indicator organisms were inoculated by cross-streaking them perpendicularly to the line of the producer inoculum. Seventeen strains of Acinetobacter (17 different serotypes of var. anitratum obtained

from CDC) (28) were tested for bacteriocin production against these same 17 strains as indicators. Only one strain, #9, demonstrated bacteriocin production active against one indicator strain, #6. It was observed that the growth characteristics of #6 were somewhat different from most of the other strains. It grew in clumps in broth media; when inoculated onto plates, it did not produce a confluent lawn but grew sparsely.

Modifications of the original procedure included using all combinations of either HI agar or BHI agar for growth of producers and either TSB or BHI broth for growth of the indicators to be used in the cross-streaking. Under these varied conditions, #9 was still the only strain demonstrating bacteriocin production, and #6 was the only strain that was susceptible.

2. Agar Overlay Method

Potential producer strains were inoculated onto nutrient agar surfaces as "spots" of organisms to be tested. Following overnight incubation, the growth was exposed to chloroform vapours and the indicator was applied in an overlay of soft agar (final concentration was approximately 10^6 bacteria/ml).

Sixty-five strains of Acinetobacter were tested for their ability to produce bacteriocins against these 65, plus 5 additional Acinetobacter strains, as indicators. Only 11 strains demonstrated production of bacteriocins that were active against 9 susceptible strains as shown in Table 2.

The degree of activity, observed as differences in zone sizes, varied. Some zones surrounding the producer "spots" were approximately 1 mm wide, whereas others were in excess of 5 mm. Also, the numbers of resistant colonies within the zones of inhibition varied from zero to greater that 100.

Some bacteriocin activity was detected using the agar methods; however, the number of strains demonstrating bacteriocin production and/or susceptibility was small.

Table 2. Strains of Acinetobacter showing bacteriocin production and susceptibility when 70 strains were tested by the agar overlay method

		Indicators								
		6	18	26	33	37	38	39	49	51
Producers	2	x			x				x	
	9	x			x	x			x	
	28		x							
	29			x						
	35						x	x		
	37			x						
	46	x								
	49					x				
	52							x		x
	54	x							x	
	65	x								

B. Broth Media

1. Mitomycin Induction

The potential producer organism grown in TSB overnight at 32 C was inoculated into TSB to obtain a final concentration of 10^7 bacteria/ml. After 1 hour incubation at 32 C, 1 ml of 25 ug/ml mitomycin C was added. The preparation was incubated for another 5 hours, chloroformed and dropped onto indicator lawns prepared with 10^5 bacteria/ml in an aqueous agar overlay.

The 17 different serotypes of Acinetobacter var. anitratum were tested as producers against the same 17 strains as indicators. All 17 strains demonstrated some bateriocin production and 5 strains were susceptible as shown in Table 3. The degree of production and/or susceptibility was very low. Six of the 17 producers did not demonstrate greater that H activity against any of the 5 indicators. As mentioned previously, H was defined as inhibition greater than seen with the control, but with more than 100 colonies within the zone of inhibition. However, it was difficult to determine whether the clearing of the lawn observed with the bacteriocin preparations was greater than that sometimes produced by the control broth alone. Also, 4 of the 5 indicators appeared susceptible to their own bacteriocins. This phenomenon occurs rarely, as bacteriocinogenic cells are generally immune to the specific bacteriocins which they are capable of producing (80). Aside from #6 as an indi-

cator, only 2 other strains showed greater than H susceptibility. Only 6 producers produced greater than H activity against strains other than #6.

Table 3. Strains of Acinetobacter showing bacteriocin production and susceptibility when 17 strains were tested by the mitomycin induction method[a]

Producers	Indicators 6	12	13	18	19
1	H	H	H	-	-
2	1	H	H	H	-
3	H	H	H	H	-
4	1	H	H	H	-
5	1	H	1	H	-
6	1	H	H	-	-
7	H	1	H	H	-
8	1	1	H	H	H
9	1	H	H	H	H
11	1	H	H	H	H
12	H	H	H	H	H
13	H	-	H	H	-
15	2	H	1	H	H
16	H	H	H	H	H
17	H	H	H	H	H
18	H	H	1	H	H
19	H	H	1	H	-
C[b]	-	-	-	-	-

[a]H = questionable inhibition; 1 = 50 to 100 colonies within the zone of inhibition; 2 = 10 to 50 colonies; 3 = 1 to 10 colonies; 4 = complete clearing; C[b] = control

The above method was carried out with two modifications: (1) incubating the bacteriocin producing organisms at 35 C; (2) preparing the indicator lawn by seeding 3 ml aqueous agar with approximately 10^5 bacteria/ml of a log phase suspension of organisms. Previously, approximately 10^6 bacteria/ml of a stationary phase suspension was used.

When indicator lawns were prepared with a smaller number of bacteria, activity appeared considerably greater, as shown in Table 4. However, it was still difficult, in some cases to distinguish clearing which was due to bacteriocin preparations from that of the broth control. Two strains, #7 and #12, appeared susceptible to the bacteriocins which they produced.

Table 4. Bacteriocin production and susceptibility of Acinetobacter using the mitomycin induction method when indicator lawns were prepared with 10^5 bacteria/ml[a]

		Indicators						
		5	6	7	8	9	11	12
Producers	5	-	H	H	-	-	H	H
	6	-	CN[b]	-	-	-	-	CN
	7	H	3	1	H	1	-	H
	8	1	3	-	-	1	H	H
	9	3	3	H	-	-	H	3
	12	H	3	1	-	1	-	H

[a]see legend to Table 3; [b]contamination

Different concentrations of mitomycin C, 0, 1, and 5 ug/ml were used in broths for bacteriocin production at 32 C by strain #5 and #6. The indicator lawn was prepared by inoculating a log phase suspension of organisms into aqueous agar at a concentration of 10^5 bacteria/ml and overlayed onto a BHI agar plate. No activity was observed against indicator strains #5, 6, 7, and 8.

Acinetobacter strains #5 and 9 were tested against indicator strains #5, 6, 7, 8, and 11 by increasing the concentration of producer organisms from 10^7 to 10^8 bacteria/ml and incubating at 35 C. No activity was demonstrated.

In another modification, the indicator bacteria were flooded onto the surface of a TSA plate at a concentration of 10^4 bacteria/ml and the mitomycin C induced bacteriocin preparations were dropped onto it. Two strains, #9 and 46, were tested for bacteriocin production against strains #2, 12, 19, 38, and 49. No activity was observed.

This method of mitomycin C induction, originally developed by Farmer for testing Serratia marcescens (49, 50) did not give clearcut results when testing for bacteriocin production by Acinetobacter. Even when various modifications of the original method were tried, little or no activity was observed, and interpretation of zones of inhibition of potential producers compared to controls was difficult.

2. Mitomycin Induction with Aeration

A TSB suspension prepared with cell paste of producer organism was diluted in TSB to give a final concentration

of 10^8 bacteria/ml. Following 2 hours incubation in a 32 C shaking water bath, 1 ml of 30 ug/ml mitomycin C was added and incubation with shaking was continued for another 5 hours. The preparations were chloroformed and dropped onto indicator lawns that had been prepared by flooding TSA plates with a TSB suspension of indicator organisms containing 10^4 bacteria/ml.

Six Acinetobacter strains were tested as producers against 34 strains as indicators. Four of the strains demonstrated slight bacteriocin activity against 12 of 34 indicators as shown in Table 5; however, the activity was not strong. The results were easier to read compared to the previous method of mitomycin C induction without aeration. The control broths did not "mark" the lawns as much, and consequently comparisons between the test preparations and the controls were less confusing. Because of the low degree of activity, however, this was a relatively insensitive method for detecting bacteriocin activity of Acinetobacter.

Although some Acinetobacter strains demonstrated bacteriocin activity by the agar methods, and some by the broth methods, there was not sufficient bacteriocin production and susceptibility to develop a typing system.

There are several possible explanations for the low degree of observable activity.

Table 5. Bacteriocin production of Acinetobacter using the mitomycin induction-aeration method a

Indicators

Producers	1	2	3	4	5	6	7	8	9	11	12	13	15	16	17	18	19	20	21	23	28	32	33	37	38	39	42	46	49	50	51	54	56	58
9	-	-	-	-	1	-	-	-	-	-	1	-	-	-	1	-	-	-	-	1	-	-	-	1	1	2	-	1	-	-	1	1	1	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	1	1	-	-
46	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	1	-	1	-	-	-	2	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	-	1	-	1	1	2	-	1	-	-	1	1	-	-
54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]see legend to Table 3

One possibility is that the proper conditions for bacteriocin production by Acinetobacter were not found. Bacteriocin production represents a lethal event for the producing cell. It is not a spontaneous occurrence and it is a condition that is repressed in most cells under ordinary circumstances (54). However, bacteriocin production can often be induced by irradiation or treatment with certain chemicals, such as mitomycin C. In the agar methods, no induction was used, whereas varying concentrations of mitomycin C were used in the broth methods. Although activity appeared to be influenced by the addition of mitomycin C, interpretation of results was still difficult. This suggested that the yield of bacteriocin was low. Induction by exposure to x-rays or ultraviolet light might have a greater effect on production.

Even though a bacterium has the genetic ability to produce a bacteriocin, it may not do so all the time under all conditions (57). The environmental conditions most suitable for bacteriocin production by a particular organism must be determined empirically, and the conditions most suitable for bacteriocinogenesis by one organism may inhibit this process in another (56). There are several variables to consider. The composition of the media used is of utmost importance, as certain nutrients and trace elements may be required for bacteriocin production (75). Several investigators have found that the composition of the media has a great effect on bacteriocin production by various Strepto-

coccus species (81, 82). It has been shown with E. coli that colicin production varied considerably when different batches of media were used (56). The pH of the media is also an important consideration. The length and temperature of incubation may also affect bacteriocin production. Wahba has shown that the amount of pyocin produced at 32 C was considerably greater than at 35 C (55).

Alternatively, bacteriocins may have been produced but inactivators might also have been produced by either the producer or the indicator organisms. If this were the case, the bacteriocins would have been inactivated before they had a chance to reach the susceptible cells. Such a phenomenon has been shown to occur with Pseudomonas aeruginosa (55), Serratia (83), and E. coli (84). Quite often, the effects of these inactivators can be eliminated by altering the environmental conditions.

In both the agar and broth methods, chloroform was used to kill viable producer bacteria. Some bacteriocins have been shown to be inactivated by chloroform (77). Since alternative methods of sterilization were not tested, the possibility that chloroform-sensitive bacteriocins were produced cannot be excluded.

Another possible explanation for the low degree of observable bacteriocin activity among the Acinetobacter strains may reflect the inadequacy of the indicator systems tested. Bacteriocin killing is dependent upon specific receptors on the surface of susceptible cells. If such susceptible cells

were not available among the Acinetobacter strains, bacteriocin activity would not be observed. Also, a suitable environment is necessary for the reaction of bacteriocin and susceptible cells.

Even though bacteriocin production by Acinetobacter was not sufficient to develop a typing system of Acinetobacter indicators, the possibility was considered that bacteriocins produced by related genera might demonstrate greater activity against Acinetobacter. Since Pseudomonas aeruginosa is considerably closely related to Acinetobacter on the phylogenetic scale (62, 63), and since methods of pyocin production have been developed, pyocin activity against Acinetobacter was investigated.

V. Evaluation of Bacteriocin Production and Susceptibility -- Pseudomonas aeruginosa against Acinetobacter

A. Agar Overlay Method

The potential pyocin producing Pseudomonas aeruginosa strains were spotted onto nutrient agar plates. Following overnight incubation, the growth was chloroformed and overlayed with soft agar that had been seeded with the Acinetobacter indicators. In early experiments, the agar overlay contained 10^6 indicator bacteria/ml. However, more reproducible results were obtained when the concentration was adjusted to 10^5 bacteria/ml of soft agar.

When 11 strains of Pseudomonas aeruginosa were tested as pyocin producers against 60 strains of Acinetobacter, 10

strains demonstrated activity against 21 strains of Acinetobacter. Three strains of Pseudomonas maltophilia were also tested for bacteriocin production against these 60 Acinetobacter strains and all were negative. The 21 strains of Acinetobacter that were susceptible to pyocins were then tested against 15 additional strains of Pseudomonas aeruginosa. Ten of the 15 Pseudomonas aeruginosa strains exhibited pyocin production that was active against all 21 Acinetobacter indicator strains.

The zones produced by pyocin inhibition varied in size. Some were only 1 mm beyond the edge of the spot of producer growth whereas others were more that 15 mm. These large zones often were superimposed on the zones of adjacent producer organisms. In these cases, it was necessary to repeat the test, applying fewer producer organisms per plate and spacing them further apart.

B. Broth Media

1. Mitomycin Induction

The pyocin producer organism, grown overnight in TSB at 32 C, was inoculated into TSB to obtain a final concentration of 10^7 bacteria/ml. After 1 hour incubation at 32 C, 1 ml of 25 ug/ml mitomycin C was added. The preparation was incubated for an additional 5 hours, chloroformed, and dropped onto indicator lawns prepared by flooding TSA plates with TSB suspensions of indicator Acinetobacter strains containing 10^4 bacteria/ ml.

Four potential producers (2 which demonstrated activity against Acinetobacter by the agar method and 2 that did not) were tested against 5 strains of Acinetobacter and 2 strains of Pseudomonas aeruginosa as indicators. No activity was observed against the Acinetobacter strains as shown in Table 6. Both strains of Pseudomonas aeruginosa indicators were susceptible to 3 of the 4 pyocins.

Table 6. Pyocin activity against Acinetobacter and Pseudomonas aeruginosa using the mitomycin induction method [a]

		Indicators						
		Acinetobacter					Ps. aeruginosa	
		2	12	19	38	49	102	115
Producers	101	-	-	-	-	-	-	-
	108	-	-	-	-	-	4+	4+
	112	-	-	-	-	-	3+	4+
	116	-	-	-	-	-	4+	4+
	C	-	-	-	-	-	-	-

[a] see legend to Table 3

2. Mitomycin Induction with Aeration

A TSB suspension prepared with cell paste of the pyocin producer was diluted in TSB to give a final concentration of 10^8 bacteria/ml. Following 2 hours incubation in a 32 C shaking water bath, 1 ml of 30 ug/ml mitomycin C was added and incubation with shaking was continued for another 5 hours. The preparations were chloroformed and dropped onto indicator lawns. Both agar overlay and flooding methods

were evaluated for preparation of the indicator lawns. Zone edges were sharper and there were fewer colonies within the zones when the flooding method was used. When pyocin was dropped onto an agar overlay indicator, it did not appear to diffuse through the depth of this overlay, because there were colonies remaining in the lower depths of the agar overlay but not at the surface. This made interpretation difficult. In subsequent experiments, the indicator lawn was prepared by flooding a TSA plate with a TSB suspension containing 10^4 indicator bacteria/ml.

When the susceptibility of 33 Acinetobacter strains (which included all those that were susceptible by the agar method) to 7 pyocin preparations was tested by this method, 24 strains were susceptible. Four of the pyocin preparations demonstrated activity against the Acinetobacter strains. The susceptibility of 5 strains of Pseudomonas aeruginosa to these 7 pyocin preparations was also tested. All 7 were selectively active against at least 4 of the 5 Pseudomonas aeruginosa indicators. The effects of these 7 pyocin preparations on a susceptible strain of Acinetobacter, strain number 32, and a susceptible strain of Pseudomonas aeruginosa, strain number 101, are shown in Figure 1 and Figure 2 respectively.

3. Medium 81

Pyocin producers were grown in Medium 81 (TSB without glucose plus 1% potassium nitrate) overnight in a 32 C

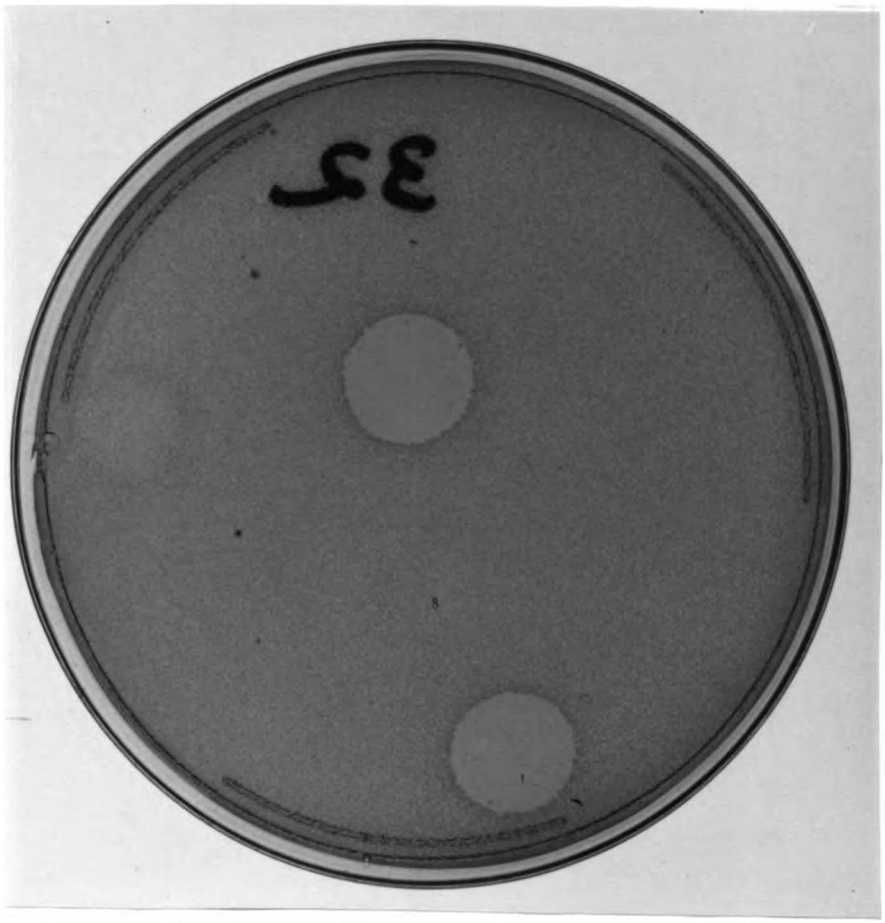


Figure 1. Pyocin susceptibility plate of Acinetobacter strain #32 to pyocins prepared by the mitomycin C induction-aeration method. Two pyocin preparations produced areas of 4+ inhibition. One preparation produced H clearing as evidenced by the faint zone at 10 o'clock.

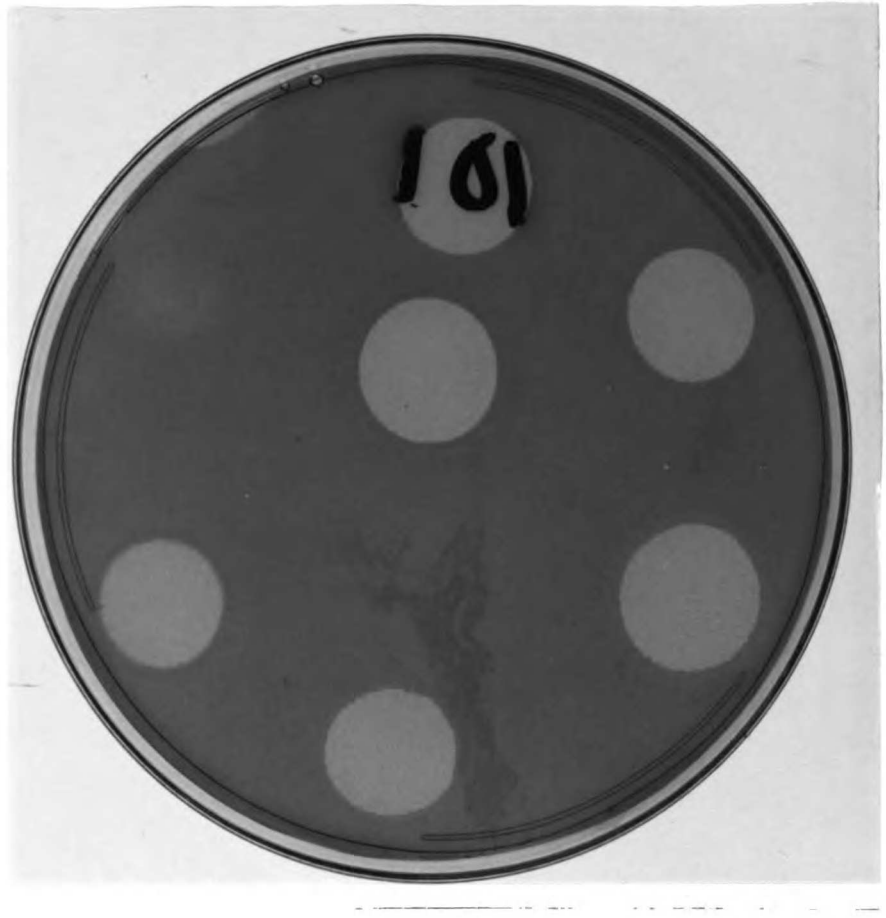


Figure 2. Pyocin susceptibility plate of Pseudomonas aeruginosa strain #101 to pyocins prepared by the mitomycin C induction-aeration method. Six pyocin preparations produced areas of 4+ inhibition. One preparation (at 11 o'clock) produced H inhibition.

water bath. After chloroforming, these preparations were dropped onto indicator lawns that were prepared by flooding TSA plates with Medium 81 suspensions containing approximately 10^4 indicator bacteria/ml.

Three pyocin preparations were tested against 8 Acinetobacter and 7 Pseudomonas aeruginosa indicator strains as shown in Table 7. Two preparations demonstrated activity against 1 Acinetobacter indicator. All pyocins were active against Pseudomonas aeruginosa. The pyocin activity against Pseudomonas aeruginosa was the same whether the indicators were prepared in Medium 81 or TSB.

Since Pseudomonas aeruginosa can utilize nitrate as a terminal electron acceptor, pyocin production in Medium 81 eliminates the need for aeration. For purposes of typing, sufficient quantities of pyocin have been produced in Medium 81 without induction. This method was not evaluated for bacteriocin production by Acinetobacter, since Acinetobacter cannot use nitrate as a terminal electron acceptor and instead requires oxygen (12).

Of the 3 broth methods evaluated, the method involving mitomycin C induction and aeration produced pyocins demonstrating the greatest activity against Acinetobacter. This method was used in most of the following experiments.

Table 7. Pyocin activity against Acinetobacter and Pseudomonas aeruginosa using the Medium 81 method [a]

Producers	Indicators: Acinetobacter 12	18	19	26	37	38	49	32	Ps. aeruginosa 102	105	108	112	112[b]	115	115[b]	116	116[b]	117
108	-	-	-	-	-	-	-	2	4	3	-	-	-	4	4	-	-	3
112	-	-	-	-	-	-	-	-	2	3	1	-	-	4	4	-	-	1
116	-	-	-	-	-	-	-	2	4	3	-	-	-	4	4	-	-	3

[a] see legend to Table 3; [b] indicators prepared in TSB broth

C. Comparison of Pyocin Activity

The results of pyocin activity from 7 different Pseudomonas aeruginosa strains against 33 Acinetobacter strains are shown in Table 8. Data in the upper table were obtained by the mitomycin C induction-aeration broth method, that in the lower table by the agar overlay method.

Pyocin activity against Acinetobacter and Pseudomonas aeruginosa using agar and broth methods of production is summarized in Table 9. All mitomycin-C-induced aerated broth preparations were active against Pseudomonas aeruginosa indicators. Pyocins, #109 and 115, were not active against Pseudomonas aeruginosa indicators by the agar method and by the broth method using Medium 81. It appears that mitomycin C induction with aeration is necessary for these pyocins to demonstrate activity against Pseudomonas aeruginosa.

Four of the mitomycin-C-induced aerated broth preparations, #103, 108, 115, and 116 were active against Acinetobacter indicators. Only one of these, #103, was also active against Acinetobacter by the agar overlay method. Pyocins produced by Pseudomonas aeruginosa strain #104, 107, and 109 were active against Acinetobacter indicators only by the agar method.

Approximately 90% of Pseudomonas aeruginosa strains are capable of producing pyocins (58). The high frequency of this property, combined with the diversity in activity of the pyocins produced, has enabled pyocins to be effectively used in the typing of Pseudomonas aeruginosa for epidemio-

Table 8. Comparison of pyocin activity against Acinetobacter and Pseudomonas aeruginosa using; a) the Mitomycin induction-aeration broth method, and b) the agar overlay method[a]
([a]see legend to Table 3)

Indicators

a) Mitomycin induction-aeration broth method

Producers	Acinetobacter																															Ps. aeruginosa				
	1	2	3	4	5	7	8	9	11	12	13	15	16	17	18	19	20	21	23	28	32	33	37	38	39	42	46	49	50	51	54	56	58	101	102	103	104	105
103	-	-	2	-	2	1	2	2	-	-	-	1	-	-	1	-	-	1	-	1	1	-	-	2	-	-	1	-	-	1	1	-	1	4	4	1	1	4
104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	4	3	0	4
107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	4	3	0	4
108	-	-	2	-	2	2	2	2	-	2	-	1	-	1	3	-	-	1	1	2	4	1	3	1	3	1	1	2	1	2	1	-	1	4	4	4	3	4
109	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	4	2	0	4
115	-	-	-	-	-	-	-	1	-	2	-	-	-	2	1	-	-	-	-	2	1	1	1	1	1	2	1	-	-	1	2	-	-	1	4	3	1	1
116	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	4	-	1	-	-	-	2	-	-	-	-	-	-	4	4	2	0	4
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

b) Agar overlay method

Producers	1	2	3	4	5	7	8	9	11	12	13	15	16	17	18	19	20	21	23	28	32	33	37	38	39	42	46	49	50	51	54	56	58	101	102	103	104	105
103	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	-	+	-	-	-	-	+	+	-	-	-	+
104	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+
107	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+
108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
109	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
116	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+

Table 9. Summary of pyocin activity against Acinetobacter and Pseudomonas aeruginosa using various methods of production.

	Activity against Acinetobacter			Activity against Ps. aeruginosa			
pyocin	agar method[a]	broth[b]	broth[c]	agar method	broth[b]	broth[c]	Med. 81[d]
103	+	+	NT[e]	+	+	NT	+
104	+	-	NT	+	+	NT	+
107	+	-	NT	+	+	NT	+
108	-	+	-	+	+	+	+
109	+	-	NT	-	+	NT	-
115	-	+	NT	-	+	NT	-
116	-	+	-	+	+	+	+

[a]agar overlay; [b]mitomycin C induction with aeration; [c]mitomycin C induction without aeration; [d]Medium 81 (without induction or aeration); [e]not tested

logical purposes (63). Certain genera and species of organisms other than Pseudomonas aeruginosa have been shown to be susceptible to pyocins. These include E. coli (65) and the fluorescent pseudomonads: Pseudomonas putida and Pseudomonas fluorescens (61). Other organisms tested which failed to demonstrate susceptibility to pyocins include: Ps. acidovorans, Ps. cepacia, Ps. maltophilia, Ps. mendocina, Ps. pseudoalkaligenes, Ps. putrefaciens, and Ps. stutzeri (61). Even within the genus, there is preferential action against strains of the producing species. Hamon has shown that Ps. fluorescens was considerably less susceptible to pyocins than Pseudomonas aeruginosa (64). Pyocin activity has not been reported to be used in typing organisms other than Pseudomonas aeruginosa.

Approximately 1/3 of the Acinetobacter strains tested by the agar method were susceptible to pyocins. Even though induction was not used, large quantities of pyocin appeared to be produced as evidenced by the wide zones of inhibition of both the susceptible Acinetobacter and Pseudomonas aeruginosa indicators. This indicated a high degree of diffusibility and probably a low molecular weight of the pyocin molecules.

When the Acinetobacter strains, that were susceptible to pyocins produced by the agar method, were tested for their susceptibility to pyocins produced by the broth method, only half of the strains were susceptible, and the patterns of

susceptibility were different from those produced by the agar method (Table 8). Several Pseudomonas aeruginosa indicators were also tested for their susceptibility to these pyocins. Although there were some differences in the results between the two methods, they were not as dramatic as those using Acinetobacter indicators.

Pyocin activity against the Acinetobacter indicators differed considerably between the two methods. The reasons for these discrepancies are not known, however, a few relevant factors must be considered.

With some organisms, bacteriocin production has been shown to occur on agar-solidified media but not in broth media or vice versa (56, 77). This is not surprising since the nutritional factors and cultural conditions under which the producers are grown can have a great influence on bacteriocin production. There were numerous differences in the conditions under which the producers were grown in the two methods used in this study for the evaluation of pyocin production against Acinetobacter.

Of particular significance in the agar method was the requirement for diffusion of pyocin molecules into the agar in order for activity to be observed. There was no need for pyocin produced in broth to diffuse, since each homogeneous preparation was dropped directly onto freshly inoculated indicator lawns. The increased concentration of divalent cations in the agar media may also have had an influence on pyocin production.

The length of incubation of the producers differed. In the agar method, producers were grown on agar surfaces for approximately 18 hours, reaching a stationary phase in their growth cycle prior to chloroforming. In the broth method, mitomycin C was added to a log phase growth of indicator organisms and incubation was continued for a few more hours to allow mitomycin to exert its effect. The amount of pyocin produced increases along with the number of producing organisms, hence increases with time as the bacteria multiply (76). Still, it is difficult to compare the broth and the agar methods of production with regard to the time when bacteriocin was produced because of the effect of induction in the broth method, as well as the differences in numbers of organisms, involved in each method.

Using the scrape-and-streak method, Wahba (55) has shown that the production of inactivators of pyocin reaches its peak at certain times in the growth cycle of the producer organisms. The greatest antagonistic effect occurred between 24 and 48 hours of incubation of the producers. Wahba did not study this phenomenon at less than 16 hours of incubation. The possibility exists that these or other inactivators can be produced earlier in the growth cycle. Also, induction might have a considerable effect on the production of inactivators as well as pyocins.

Bacteriocin yields often can be increased from several to a thousand-fold by induction (56). The addition of mitomycin causes a lysis of cells with release of pyocin. Ikeda

et. al. (85) have shown a correlation between an increase in pyocin activity with a decrease in the number of viable cells in the pyocin producing culture.

In this study, it was shown that the production of pyocin by strain numbers 109 and 115 was dependent upon induction. Other investigators have shown that many mucoid strains of Pseudomonas aeruginosa will produce pyocin only upon induction with mitomycin C (86). Although strain numbers 109 and 115 did not appear mucoid in colonial morphology, they failed to produce detectable pyocin by the agar overlay method, a method without induction. Since induction was incorporated into the broth method only, this, too, may contribute in part to some of the discrepant results.

In the agar method, pyocin producers were incubated at 35 C, and in the broth method they were incubated at 32 C. Gilles and Govan (39), using the scrape-and-streak method, have shown that temperature of incubation did have an effect on pyocin production, greater pyocin production occurring at 32 C than at 35 C.

Pyocins are a heterogeneous group of substances differing in molecular size and structure, sensitivity to proteolytic enzymes, and thermal stability (58, 75, 76). Some strains of Pseudomonas aeruginosa have been shown to produce more than one pyocin (58). Ito, et. al. (87) have found one strain of Pseudomonas aeruginosa that produces both S and R type pyocins. According to Bradley (75), the S type are

characterized by their sensitivity to proteolytic enzymes and a lack of structure in electron microscopy. The R type are resistant to proteolytic enzymes and resemble phage components in appearance. It is possible that a Pseudomonas aeruginosa strain that is capable of producing more than one pyocin might produce each under different environmental conditions.

Susceptibility to pyocins may vary under different environmental conditions. Because the different methods resulted in greater variations in pyocin activity against Acinetobacter than against Pseudomonas aeruginosa, it is possible that indicator organisms of a genus and species different from the producer (and less susceptible to pyocins overall) are more sensitive to these changes.

As discussed, this work has shown that certain strains of Pseudomonas aeruginosa may produce pyocin by one method but not by another and that certain strains may produce more than one pyocin. Also, the susceptibility of the indicators to pyocin may differ under various environmental conditions.

In the following experiments, the activity of pyocin against Acinetobacter and Pseudomonas aeruginosa will be compared with respect to their physical and chemical characteristics and kinetics of killing.

VI. Assessment of Pyocin Activity

A. Characteristics of the 7 Pyocin-Producing Pseudomonas aeruginosa Strains

The 7 pyocin-producing Pseudomonas aeruginosa strains were tested for their serotype and pyocin type. These results are shown in Table 10.

Table 10. Serotype and pyocin type of the Pseudomonas aeruginosa strains used as pyocin producers in this study.

Ps. aeruginosa strain	Serotype[a]	Pyocin type[b]
103	9	626684
104	10	421421
107	7,8	111124
108	11	611131
109	9,14	non-typable
115	1,9,10	non-typable
116	11	611131

[a]Agglutination reactions using antisera (Difco) prepared against 17 different serotypes of Pseudomonas aeruginosa (73)

[b]Pyocin production patterns against eighteen standard Pseudomonas aeruginosa pyocin indicators ALA 1 - ALA 18 (26)

Some of the Pseudomonas aeruginosa strains agglutinated in more than one of the 17 different antisera, indicating that they possessed antigens against more than one serotype.

Although Pseudomonas aeruginosa strains #108 and 116 were of the same serotype and pyocin type, their activity against Acinetobacter indicator strains differed. Also, their gross phenotypic characteristics were different.

Table 11. Titers of pyocin against Acinetobacter and Pseudomonas aeruginosa using both chloroform and filtration methods of sterilization

Pyocins	Indicators: Acinetobacter(#32)	Indicators: Ps. aeruginosa(#101)
103 chloro[a]	1	10,000
103 M.P.[b]	< 1	100
104 chloro	-	1,000
104 M.P.	-	10
107 chloro	-	100,000
107 M.P.	-	10
108 chloro	1,000	100,000
108 M.P.	1	10
109 chloro	-	10
109 M.P.	-	1
115 chloro	-	1
115 M.P.	-	< 1
116 chloro	1,000	100,000
116 M.P.	10	1,000

[a] pyocin preparation sterilized by chloroforming

[b] pyocin preparation sterilized by millipore filtration

B. Titers

Pyocin preparations produced by the mitomycin C induction-aeration method and sterilized by either chloroforming or filtration, were diluted and spotted onto indicator lawns of a susceptible Pseudomonas aeruginosa or Acinetobacter indicator. The titer was defined as the reciprocal of the highest dilution demonstrating 2+ or greater activity. The titers are shown in Table 11.

A comparison of the titers of pyocin produced by the mitomycin C induction-aeration method and the Medium 81 method are shown in Table 12. The titers produced using the mitomycin C induction-aeration method were considerably higher than those using Medium 81 (without induction or aeration.

Table 12. Comparison of titers of pyocin activity against Acinetobacter and Pseudomonas aeruginosa using the mitomycin induction-aeration method and the Medium 81 method.

		Indicators	
		Acinetobacter	Ps. aeruginosa
Pyocins	10^8 mitomycin-aeration	1000	100,000
	10^8 Medium 81	1	1

Titers of pyocin against Acinetobacter were lower than those against Pseudomonas aeruginosa, indicating that Pseudo-

monas aeruginosa was more susceptible to pyocin than Acinetobacter. Pseudomonas aeruginosa strain #108 was susceptible to chloroform-sterilized pyocin preparations #108 and #116 up to a dilution of 1:100,000, whereas Acinetobacter strain #32 was only susceptible up to a dilution of 1:1,000. When these same preparations were sterilized by filtration, Pseudomonas aeruginosa strain #101 was susceptible to pyocin #108 up to a dilution of 1:10 and to pyocin #116 up to a dilution of 1:1,000. A reduction in the susceptibility of Acinetobacter to these filtered pyocin preparations also was observed as shown in Table 11.

Only 3 of the 7 pyocin preparations were active against Acinetobacter strain #32. The activity of chloroform-sterilized pyocin preparation #103 was not strong against Acinetobacter, even though it showed a titer of 10,000 against Pseudomonas aeruginosa. Chloroform-sterilized pyocin preparation #107 produced a titer of 100,000 against Pseudomonas aeruginosa but did not demonstrate activity against any of the Acinetobacter strains by the broth method. On the other hand, pyocin preparation #115, which produced a very low titer against Pseudomonas aeruginosa, was active against several of the Acinetobacter indicators. Thus, the susceptibility of the Acinetobacter strains to pyocin was not always related to a high titer of activity against Pseudomonas aeruginosa.

The titers of pyocin were consistently higher when sterilization was by chloroforming rather than by filtration. The procedure for filtration involved centrifugation of the preparation and passing the supernatant through a millipore filter. It is possible that much of the pyocin remained associated with the cells and was carried into the sediment. The procedure for chloroform sterilization involved adding chloroform, then agitating the entire preparation prior to centrifugation. It appeared as if the chloroform somehow released pyocin from the producer cells. Several colicins (88) and pyocins (89) have been shown to be associated with cell wall components, specifically lipopolysaccharide. If lipids are responsible for binding bacteriocin to the producer cell, the ability of chloroform to disrupt the bacteriocin-cell relationship and release the bacteriocin seems likely.

The effect of mitomycin C induction on the production of pyocin is shown in Table 12. Titers were significantly higher against both Acinetobacter and Pseudomonas aeruginosa when induction was used. This is in agreement with previous work describing the effectiveness of mitomycin C in inducing bacteriocin production (56, 76).

Although Medium 81 has been used in a simplified method for pyocin production (26, 41) and has been useful in pyocin typing, the titers of pyocin produced are much lower than those produced when induction is used.

As mentioned previously, units of pyocin activity are defined as the titer. If the particular indicator is very susceptible to a pyocin preparation, that preparation will demonstrate a higher titer or more units of pyocin than if it were tested against a less susceptible indicator. For this reason, it is important to define the test conditions when describing titers or units of pyocin.

C. Chloroform Sensitivity

Pyocin sensitivity to chloroform was determined by preparing pyocins by the mitomycin C induction-aeration method and subjecting a portion of each preparation to chloroform sterilization and a portion to millipore filtration sterilization. With all 7 pyocins, titers against both Acinetobacter and Pseudomonas aeruginosa were higher when chloroform sterilized. Therefore, none of the 7 pyocin preparations were chloroform sensitive.

Chloroform sensitivity has been a discriminating characteristic in categorizing certain bacteriocins. Prinsloo (77) was able to define two groups of marcescins produced by Serratia marcescens based on this and a few other characteristics. Certain colicins have been shown to fit a similar pattern (90). Since the pyocins tested in this experiment were all chloroform resistant, this criterion was not beneficial in trying to explain some of the varying results.

D. Trypsin Sensitivity

One percent trypsin was added to undiluted pyocin preparations #103, 104, 107. 109, and 115 and to undiluted

and 10-fold (to 10^{-6}) dilutions of pyocin preparations #108 and 116 to give a final concentration of 330 ug/ml trypsin. Following incubation at 37 C for one hour, the pyocin preparations were dropped onto indicator lawns of both Acinetobacter and Pseudomonas aeruginosa. No reduction in activity was observed following trypsin treatment; therefore, all 7 pyocin preparations were trypsin resistant.

Previous studies have shown that pyocins (55) and other bacteriocins (56, 75, 77) varied in their susceptibility to the inactivating action of trypsin (at concentrations equal to or less than that used in this study). Trypsin did not inactivate any of the 7 preparations tested here, and this did not help to differentiate any of these pyocins.

E. Thermal Stability

The thermal stability of the 7 pyocin preparations was tested by heating aliquots of pyocin prepared by the mitomycin C induction-aeration method for 30 minutes at 50 C, 60 C, or 70 C. The pyocins were dropped onto lawns of both Pseudomonas aeruginosa and Acinetobacter indicator strains. Pyocins were defined as heat stable when no loss of activity against either Acinetobacter or Pseudomonas aeruginosa occurred. The results are shown in Table 13.

Table 13. Thermal stability of pyocin activity

Pyocins	Temperature 50 C	60 C	70 C
103	+[a]	-[b]	-
104	+	+	-
107	+	+	-
108	+	-	-
109	+	+	-
115	+	-	-
116	+	-	-

[a]heat stable

[b]heat labile

The heat stability of bacteriocins has been shown to differ considerably (56). In this study, all of the pyocin preparations were stable at 50 C and labile at 70 C. However, varied stability was detected at 60 C. The four preparations that were inactivated at 60 C were those that demonstrated activity against Acinetobacter indicators by the broth method. This observation supports the notion that there is something distinctive about those pyocins that are active against Acinetobacter by the broth method. It is possible that a more detailed characterization would demonstrate other similarities and differences between the two groups of pyocins.

VII. Kinetic Studies

The kinetics of pyocin killing of both Acinetobacter and Pseudomonas aeruginosa were examined.

The pyocin used in this experiment was produced by Pseudomonas aeruginosa strain #108 using the mitomycin C induction-aeration method. The pyocin-susceptible Acinetobacter strain #32 and Pseudomonas aeruginosa strain #101 were used as indicators.

Both indicator strains were grown in TSB overnight at 35 C. The numbers of viable organisms contained in each suspension and respective dilutions were determined by plate counts.

The overnight TSB suspension of Pseudomonas aeruginosa strain #101 was diluted to 10^{-3} in both TSB and saline. The overnight suspension of Acinetobacter strain #32 was diluted in TSB, saline, MHB, TSB-Ca^{++}, and MHB-Ca^{++}. At time zero, 0.1 ml of undiluted pyocin was added to each bacterial suspension. For Pseudomonas aeruginosa, this corresponded to 9 x 10^5 bacteria/ml and for Acinetobacter, to 6 x 10^5 bacteria/ml. At various times after addition of pyocin to the susceptible bacteria, samples were removed from the reaction vessel and diluted to stop bacteriocin adsorption. From these dilutions, viable counts were done in order to determine the rate at which bacteria were killed by pyocin. The rates of pyocin killing of Acinetobacter and Pseudomonas aeruginosa can be demonstrated by plotting the number

of surviving bacteria in respect to time, as shown in Figures 3 and 4.

With Pseudomonas aeruginosa, there was a 2 log reduction in the number of bacteria within 30 seconds following the addition of pyocin. With Acinetobacter, a similar degree of killing took nearly 4 hours.

The more rapid killing of Pseudomonas aeruginosa as compared to Acinetobacter could involve differences in either the adsorptive or lethal phases of bacteriocin action.

The adsorptive phase involves the adsorption of bacteriocin molecules to specific receptors on the surface of susceptible cells. The number of receptors on susceptible cells can vary among a population of bacteria and may be affected by the medium in which the bacteria are suspended (56). Bacteriocin killing has been shown to follow single hit kinetics, whereby bacteriocin action can progress to the lethal phase following the effective adsorption of a single bacteriocin molecule. However, only a proportion of the receptors are able to take part in effective adsorption; that is adsorption leading to cell death (58). The number of molecules which have to be adsorbed in order to kill a susceptible cell is referred to as a lethal unit. The number of molecules that constitute a lethal unit depends on; the bacteriocin preparation, the susceptible bacteria, and the test conditions (56).

Figure 3: Pyocin killing of Acinetobacter calcoaceticus and Pseudomonas aeruginosa suspended in TSB and saline. Samples for viable counts were taken at the indicated times. Symbols: (▲) pyocin versus Acinetobacter-saline; (●) pyocin versus Acinetobacter-TSB; (■) pyocin versus Pseudomonas aeruginosa-saline: (Δ) pyocin versus Pseudomonas aeruginosa-TSB.

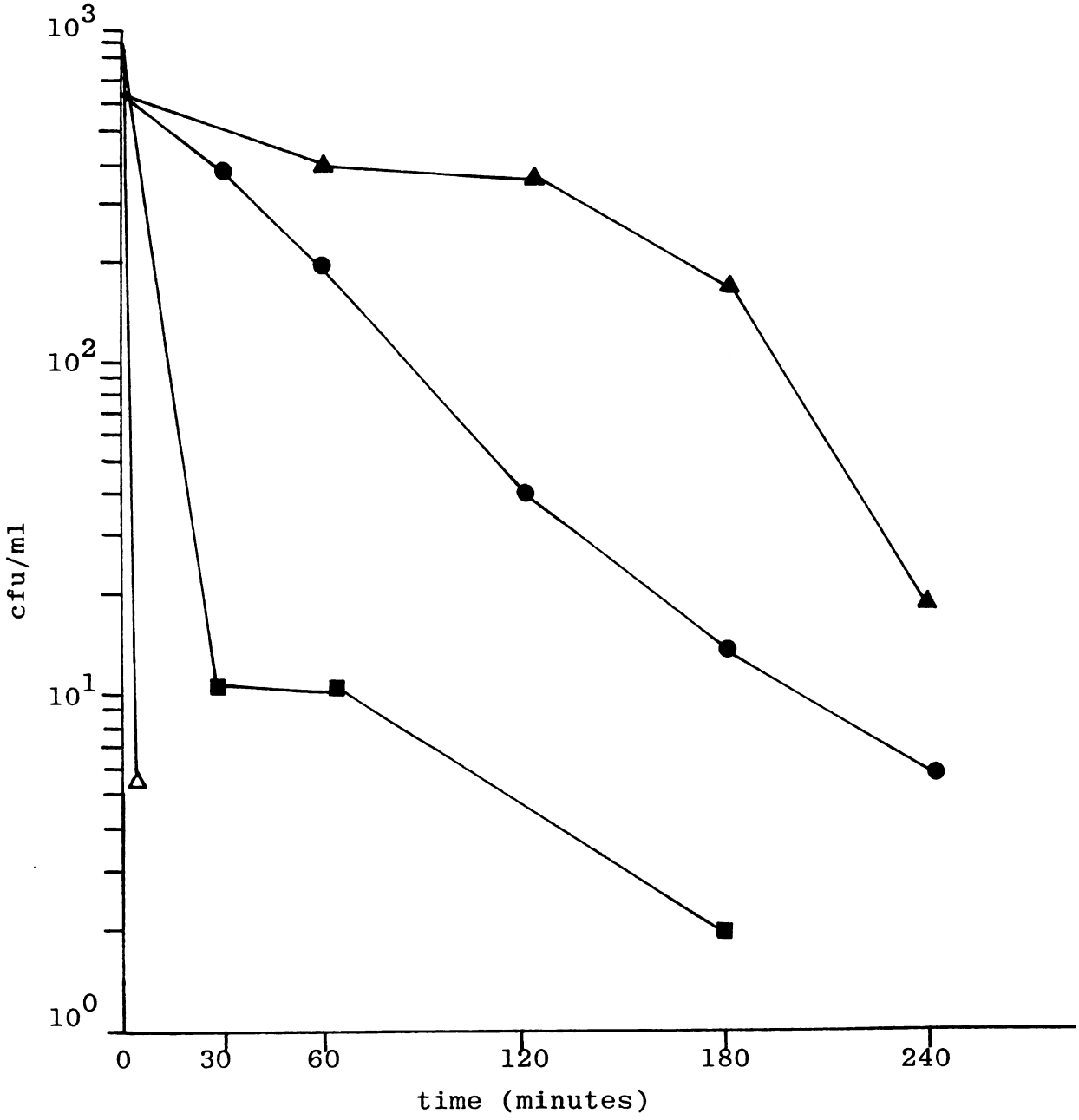
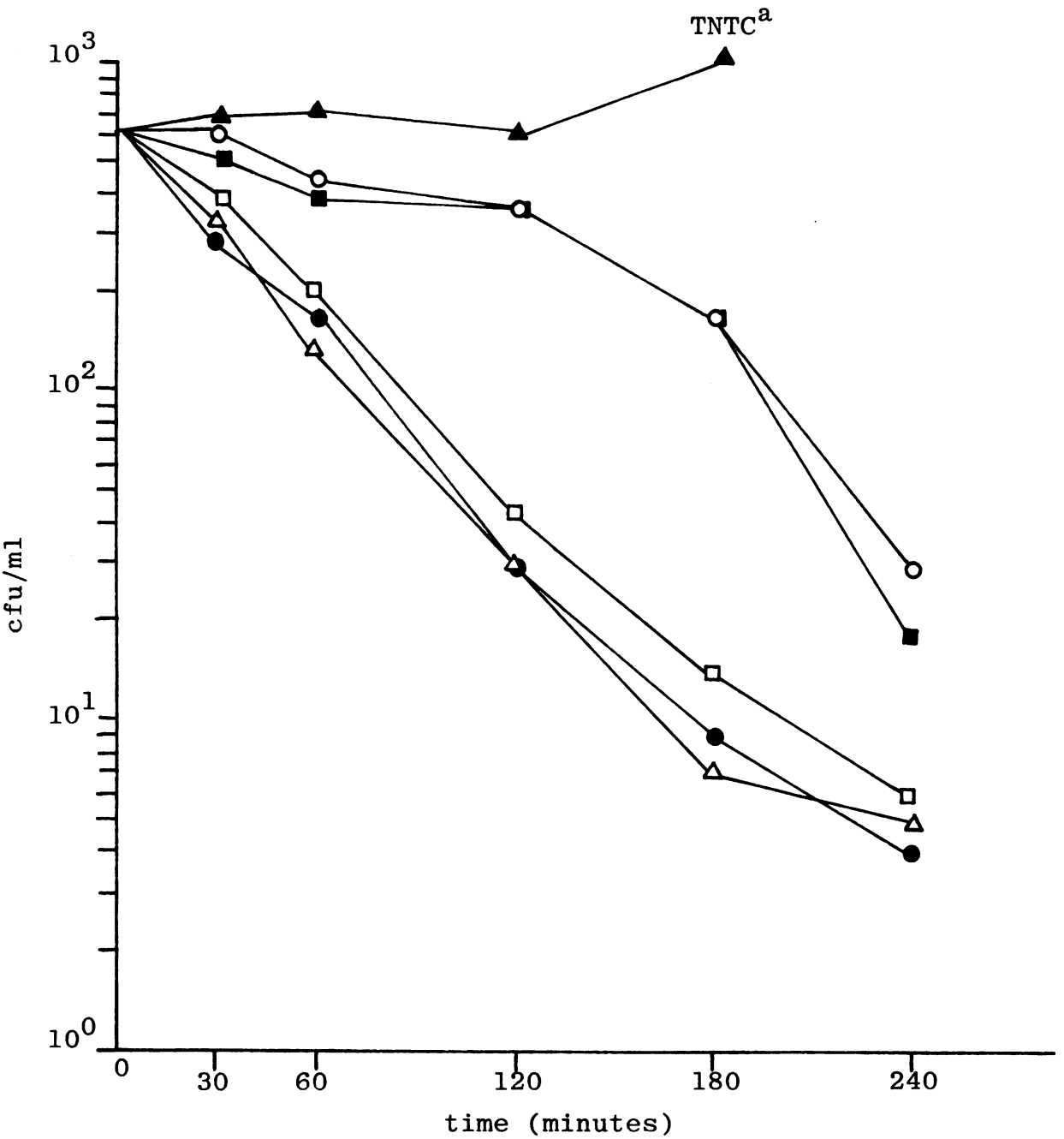


Figure 3.

Figure 4. Pyocin killing of Acinetobacter calcoaceticus suspended in various media. Samples for viable counts were taken at the indicated times. Symbols: (▲) control, untreated TSB suspension; (○) MHB: (■) saline; (□) TSB; (△) TSB-Ca^{++}; (●) MHB-Ca^{++}.



[a]too numerous to count

Figure 4.

It is probable that the susceptible Pseudomonas aeruginosa strain (#101) used in this experiment had more receptors for the pyocin molecules than did the Acinetobacter strain (#32). Also, the number of pyocin molecules required to kill a Pseudomonas aeruginosa bacterium (the number of molecules in a lethal unit) may be fewer than the number of molecules required to kill an Acinetobacter bacterium. Both of these explanations could account for the slower killing of a population of susceptible Acinetobacter compared to Pseudomonas aeruginosa.

The events leading to the lethal phase, following adsorption, vary among the different bacteriocins. Most bacteriocins have been shown to remain at the receptor site and affect the target by a specific stimulus sent through a specific transmission system, presumably in the cytoplasmic membrane (91, 92, 93). The ultimate lesion varies. Certain bacteriocins affect DNA metabolism (92, 94), others affect protein synthesis (92), and still others affect some aspect of energy metabolism (94,95).

In this study, the events following pyocin adsorption may have occurred more quickly with Pseudomonas aeruginosa than with Acinetobacter. The Acinetobacter strain may have been deficient in a particular requirement necessary for this process thus prolonging cell death.

Calcium appeared to facilitate the pyocin reactions. Pyocin killing of Acinetobacter in TSB, TSB-Ca^{++}, and MHB-Ca^{++}

was more rapid than in MHB (which has a much lower concentration of calcium) and saline. Although pyocin killing of Pseudomonas aeruginosa was faster in TSB than in saline, the effect of calcium was less dramatic than with Acinetobacter, probably because the adverse effects produced by the lack of this ion were overcome more readily. The adsorption of bacteriocin to specific receptors on the surface of susceptible cells depends on the ionic environment (58). Certain ions are needed and certain ions may non-specifically inhibit adsorption (56). Mayr-Harting et.al. (96) have demonstrated that the concentration of Mg^{++} ions affected the lethality of pre-adsorbed colicin. Similarly, Zn^{++} is inhibitory to colicin adsorption (97). The addition of NaCl to the reaction medium has been shown to protect susceptible cells from the lethal effects of colicin (98,99).

In this study, it was shown that Acinetobacter was susceptible to pyocin; however, the rate of killing was slower than Pseudomonas aeruginosa. The composition of the medium in which the reactions occurred had an effect on the rate of killing.

SUMMARY AND CONCLUSIONS

For epidemiological purposes, it is important to have a system for comparing bacterial isolates within a genus and species in order to investigate their potential spread from a central source of contamination. Acinetobacter calcoaceticus is one of many microorganisms encountered in hospital-associated infections. Since bacteriocin typing has been successfully used in determining the relatedness of strains within other genera and species, this method of typing bacterial strains was evaluated for its usefullness in the comparison of Acinetobacter isolates.

To detect bacteriocin production by Acinetobacter, several different agar and broth methods were tried with and without mitomycin C induction. Seventy strains of Acinetobacter, most of which were clinical isolates, were evaluated. Using an agar overlay method, 11 Acinetobacter strains demonstrated bacteriocin production and 9 strains were susceptible. Various broth methods of bacteriocin production were tested; however, only slight activity was observed. Because of the small proportion of Acinetobacter strains demonstrating bacteriocin production in this study, it was not possible to use this criterion to develop a bacteriocin typing system for this bacterium.

Since methods for bacteriocin production by Pseudomonas aeruginosa have been developed and since Acinetobacter calcoaceticus is related to Pseudomonas aeruginosa on the phylogenetic scale, the susceptibility of Acinetobacter to pyocins was tested. Both agar and broth methods of pyocin production were evaluated for activity against Acinetobacter as compared to Pseudomonas aeruginosa. Approximately one-third of the 70 Acinetobacter strains tested were susceptible to pyocins; however, there were differences in susceptibility between the agar and broth methods.

Of 7 broth preparations of pyocin tested, all were active against Pseudomonas aeruginosa indicators and 4 were active against Acinetobacter indicators. Further studies were performed in order to determine if there were some distinctive characteristics about those pyocins that were active against Acinetobacter. All 7 pyocins were chloroform and trypsin resistant. All were heat stable at 50 C for 30 minutes and labile at 70 C. Those 4 preparations that were active against Acinetobacter were labile at 60 C; the other 3 were stable. Pyocin titers were 2 log dilutions higher against a susceptible Pseudomonas aeruginosa indicator than against a susceptible Acinetobacter indicator. When undiluted pyocin was added to a susceptible strain of Pseudomonas aeruginosa, there was a 2 log reduction in the number of bacteria within 30 seconds.

Similar killing of a susceptible strain of Acinetobacter took approximately 3 hours. Calcium facilitated this reaction.

A low level of susceptibility of Acinetobacter to its own bacteriocins and significant susceptibility to pyocins has been demonstrated. Such bacteriocidal activity may be used for the typing of Acinetobacter isolates.

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