BACTERIOCINS OF ERWINIA CAROTOVORA

by

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ABSTRACT

The sensitivity of Erwinia carotovora subsp. atroseptica to bacteriocins produced by strains in the common potato serogroups of E. carotovora was investigated. Bacteriocins produced by representative strains of the common serogroups had activity spectra containing strains from one to six sensitive serogroups. Similarly, indicator strains representing different serogroups showed variable sensitivity. One indicator strain was sensitive to bacteriocins produced by only one producing strain while others were sensitive to bacteriocins produced by strains in several different serogroups. Bacteriocin production in the serogroups tested was detected only from strains that were biochemically E.c. subsp. carotovora (Ecc). Strains in all four E.c. subsp. atroseptica (Eca) serogroups were bacteriocin sensitive and non-producers. Some Ecc strains were bacteriocin producers and sensitive to bacteriocins produced by strains in other serogroups. Production and sensitivity were not correlated with the frequency of distribution of the more common serogroups isolated in nature. Representative strains in the two most common serogroups (I and III) were sensitive to bacteriocins produced by representative strains in three and six serogroups respectively. Strains in some of the less common serogroups (IX, XI and XVI) were bacteriocin producers but were not sensitive to the bacteriocins produced by the representative strains tested. Thus, a role for bacteriocins in the survival of these strains in nature cannot be ruled out.

Of the 44 serogroup XI strains tested by the agar overlay technique, 31 were "typical producers", 10 were "differential producers" and only three were "non-producers". However, bacteriocin production in the

latter group could be detected after induction with Mitomycin C but not with UV light. In the five serogroups in which several strains were tested, bacteriocin production and sensitivity were serogroup rather than subspecies characteristics.

In dual culture studies the starting ratio of "typical producer" to sensitive strains of 1:1000 prevented detectable growth of the sensitive strains. By comparison a starting ratio of 100:1 with a "non-producer" strain did not prevent growth of the sensitive strains. Similar results were obtained when potato tuber discs were inoculated with varying starting ratios and the population monitored after 48 h. Thus, bacteriocin producing strains have a selective advantage when grown together *in vitro* with the bacteriocin sensitive, non-producing strains.

Bacteriocin titres were enhanced by Mitomycin C induction and partial purification. Following ammonium sulfate precipitation and ultracentrifugation (150 000 x *g* for 90 min), bacteriocin activity in the resuspended pellet was associated with particles which by transmission electron microscopy resembled contractile, bacteriophage tail-like particles. These particles (due to their molecular size) were associated with small (≈ 4 mm) clear zones of inhibition in the spot assay tests. "Bacteriocin-like" activity in the supernatant was resolved by gel filtration into three fractions with estimated molecular weights of 17 700, 29 500 and 224 000 D. The first two fractions showed large (up to 20 mm) diffuse zones of inhibition. The third fraction showed small (≈ 4 mm) clear zones of inhibition. All four fractions had similar activity spectra against representative indicator strains and were produced by all of the serogroup XI producer strains tested. Relative production differed depending on the strain. The threshold of

sensitivity displayed by the indicator strains varied with the fractions. The resuspended pellets had the highest titres which suggested that those macromolecular bacteriocins were responsible for the antagonism in *in vitro* and possibly in nature.

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INTRODUCTION

Erwinia carotovora (Jones) Bergey *et al.* is an important bacterial pathogen of potato causing the diseases soft rot and blackleg. Since chemical controls have not proven effective, current control programmes are almost exclusively based on sanitation and cultural practices (Agrios 1978). A thorough knowledge of the ecology and population dynamics of the bacteria in nature is required for this approach to be successful. One aspect of the ecology of the soft rotting erwinias deserving more attention is the ability to produce bacteriocins. The selective advantage conferred on producing strains may be a factor in explaining the prevalence of certain strains in nature. It may also represent a mechanism that could be manipulated for the purpose of biological control.

LITERATURE REVIEW

A. Bacteriocins

Gratia (1925) first described a highly specific anti-bacterial compound produced by a strain of *E. coli*. This compound named "Colicine" was active against only strains of the same species (Gratia and Fredericq 1946). A more general term "bacteriocine" was proposed by Jacob *et al.* (1953) with the discovery of similar compounds in organisms other than the coliforms. This term is now spelled "bacteriocin" without the final "e" (Mayr-Harting *et al.* 1972). Compounds with similar properties have been described as being produced by *Bacillus megaterium* de Barry (megacins), by *Clostridium* spp. Prazmowski (clostocins), by *Streptococcus* spp. Rosenbach

(streptococcins), by Staphycococcus Rosenbach (staphylococcins) and many more (Tagg et al. 1976).

Based on work done on the bacteriocins produced by E. coli, (Migula) Castellani and Chalmers, Nomura (1967) defined bacteriocins as non-replicating, bactericidal, protein-containing substances which are produced by certain strains and which are active on the same or closely related species. The requirement for having receptor sites is important in distinguishing bacteriocins from less specific antibiotics. In the latest review Tagg et al. (1976) listed the following six criteria that have been used with varying degrees of consistency in defining bacteriocins:

i. Bacteriocins have a narrow range of inhibitory activity centered about the homologous species.

ii. Bacteriocins have an essential biologically active protein moeity.

iii. Bacteriocins have a bactericidal mode of action.

iv. Bacteriocins show attachment to specific receptor sites.

v. The genetic determinant of bacteriocins is plasmid-borne (in terms of production and host immunity).

vi. Production of bacteriocins is by lethal biosynthesis.

Criteria i, ii and iii are generally applicable for many of the better characterized bacteriocins. Criteria iv and v have not been documented for many bacteriocins. Criteria vi has only been shown for inducible bacteriocins. Because there is no universally accepted definition for bacteriocins and many antibacterial compounds have not been adequately characterized, Tagg et al. (1976) suggested that

incompletely defined antagonistic substances be designated "bacteriocin-like". They reserved the term "bacteriocins" for those antibacterial compounds fulfilling at least criteria ii and iii as noted above. By this definition a wide range of heterogenous substances are included. For example Streptococcin A-FF22 (Tagg et al. 1973) with a molecular weight of 8000 D, Boticin S-5 (consisting of bacteriophage tail-like particles) with a molecular weight of 4×10^7 D produced by Clostridium botulinum Van Ermergen (Ellison and Kautter 1970) and the killer particles produced by Bacillus spp. (Ackermann 1973) are all included. The latter resemble intact bacteriophage-like particles but are not able to multiply within the sensitive cells.

Bradley (1967) classified bacteriocins into two major groups. The first consists of low molecular weight compounds, non-sedimentable by high speed centrifugation which are trypsin sensitive, heat resistant and not resolved by electron microscopy. The second group contains high molecular weight compounds having the opposite characteristics.

The majority of bacteriocins are complex molecules containing protein, lipid and carbohydrate components (Tagg et al. 1976; Ballester et al. 1980). Streptococcin STH, produced by transformable group H Streptococcus spp., is a complex molecule containing the essential protein component plus lipid and phosphate groups (Schlegel and Slade 1972). Bacteriocins produced by two Clostridium spp. appear to be primarily protein in nature (Clarke et al. 1975). Sensitivity to specific enzymes such as proteinase, lipase, etc. have been widely used to identify essential chemical components of bacteriocins. However, caution in interpretation is required because Hamon and Péron (1964)

noted that proteolytic enzyme producers frequently produced bacteriocins resistant to these enzymes. Conversely, bacteriocins produced by non-proteolytic bacteria are susceptible to proteolytic enzymes.

Since the biologically-essential component is protein, bacteriocins might be expected to be good antigens. In fact antigenic differences between four bacteriocins produced by Clostridium perfringens (Veillon and Zuber) Hauduray et al. in double diffusion tests have been reported (Tubylewicz 1970). Two pyocins with different activity spectra, isolated from Pseudomonas aeruginosa (Schroeter) Migula differ serologically (Govan 1974). Antisera against bacteriocins may be useful in bacteriocin classification, in distinguishing between components of complex bacteriocins and in eliminating bacteriocins when searching for lysogenic phage (Hamon 1956). However the failure to produce antiserum against bacteriocins produced by Staphylococcus aureus Rosenbach has been noted by Gagliano and Hinsdill (1970) and Hale and Hindsdill (1973).

The receptor sites for colicins E3-CA38 (Sabet and Schnaitman 1973) and colicin M-K12 (Braun et al. 1973, 1974; Braun and Wolff 1973) have been identified as a protein in the outer membrane. This receptor site was also shown to be the receptor site for T5 phage. The receptor sites for colicin E may also be the receptor sites for bacteriophage BF23 and Vitamin B_{12} (Buxton 1971 and Di Masi et al. 1973). In other studies the lipopolysaccharide (LPS) component has been regarded as the colicin B receptor site (Guterman and Luria 1969). Similarly the LPS component of the cell envelope was also regarded as the receptor site for pyocin R, a bacteriocin produced from Pseudomonas aeruginosa (Ikeda and Egami 1973).

Various modes of action have been proposed for the colicins. Colicins E1 and K, appear to act on the cytoplasmic membrane, inhibiting ATP production, membrane transport and permeability. The loss of K^+ ion from colicin-treated cells was noted (Broda 1979). Colicin E2 has been regarded as a DNA endonuclease which degrades DNA and inhibits cell division (Nomura 1963; Schaller and Nomura 1976) presumably after entering the cell. on the other hand, colicin E3 appears to act as an RNase clearing the 16-S ribosomal RNA and hence prevent protein synthesis. (Bowman et al. 1971; Senior and Holland 1971). With both colicins E2 and E3 degradation occured in vitro after the removal of a specific "immunity protein" which forms a tight complex with the bacteriocins. This "immunity protein" was also coded by the colicinogenic factor itself. It is not well established whether the lethal action of colicin E2 and E3 is due to direct enzymatic action or activation of some endogenous enzymes residing in the sensitive cells.

The role of bacteriocins in nature may be associated with the regulation of population dynamics. Viridins, bacteriocins of alpha-hemolytic streptococci living as a component of normal microflora of the throat, have been suggested as being responsible for resistance to infection by group A streptococci (Dajani et al. 1976). When bacteriocinogenic Staphylococcus aureus strains were inoculated with group A streptococci in mixed skin infections on hamster, the latter was eliminated or reduced significantly (Dajani and Wannamaker 1973). However, some other attempts to demonstrate that bacteriocins may play a major role in the regulation of the bacterial populations have been

unsuccessful. Kelstrup and Gibbon (1969) reported that bacteriocins probably do not play a major regulatory role in bacterial populations forming dental plaque because streptococcal bacteriocins were inactivated by the enzyme protease found in the oral cavity. Also both bacteriocin producer as well as sensitive strains have been recovered simultaneously in the same samples. The presence of matrix polysaccharide probably masks the receptor sites hence enabling the escape of sensitive strains from the bacteriocin-producing strains of Streptococcus spp. (Rogers 1974).

B. Bacteriocin Production by Plant Pathogens

Species of four genera of plant pathogenic bacteria have been reported to produce bacteriocins. However, only a few species have been characterized and purified. Vidaver (1972) reported differential bacteriocin production and sensitivity among three Pseudomonas spp. All 38 strains of P. syringae (P. syringae pv. syringae Van Hall) produced bacteriocins having a broad activity spectra. Only 55% of 24 strains of P. glycinea (P. syringae pv. glycinea Van Hall) produced bacteriocins and these had a more restricted activity spectrum than those of P. syringae. Only 8% of 39 strains of P. phaseolicola (P. syringae pv.phaseolicola (Burkholder) Young et al.) produced bacteriocins and these had the narrowest range of activity. Bacteriocin sensitivity to heat and trypsin varied so that not all conformed to Bradley's (1967) classification.

Bacteriocins produced by P. syringae strain 4-A (Haag and Vidaver 1974) and strain W-1 (Smidt and Vidaver 1981) have been purified and

characterized. Both bacteriocins appear to be similar in that they are high molecular weight bacteriophage tail-like particles which are heat sensitive and trypsin resistant. The molecular weight of syringacin 4-A was determined to be 1.6×10^7 D. Production was enhanced by UV light or Mitomycin C and the product sedimented as a single component in ultracentrifugation. The major composition of syringacin 4-A was protein. Vidaver (1976) reported that when soybean seeds were treated with syringacin 4-A, prior to challenge inoculation with P. glycinea the control (without bacteriocin protection) had 20% less germination. Lesion counts on bean leaves were also significantly reduced when purified syringacin 4-A was sprayed prior to challenge inoculation with P. phaseolicola. Whether this treatment will be successful on a field scale has yet to be determined.

Bacteriocin production by P. solanacearum (Smith) Smith has also been reported by Cuppels et al. (1978). The bacteriocin was isolated and characterized from an avirulent form of strain K-60 named strain B1 Sensitivity to this bacteriocin was noted in 43 of 51 indicator strains. Bacteriocin production was enhanced ten fold by exposure to UV light but Mitomycin C (at a final concentration of 1 mg/ml or other chemical reagents (chloramphenicol or trimethorprim) caused only a doubling. The optimal temperature for bacteriocin production was recorded as 30° C whereas the optimal growth temperature of the bacterium was 32° C. The bacteriocin had an approximate molecular weight of 65 000 D, was sedimentable and resistant to enzymes. These characteristics conformed to Bradley's (1967) low molecular weight group. (Chen and Echandi 1981) reported that seedling roots soaked in

suspensions of an avirulent, bacteriocin-producing strain conferred a high degree of protection against the virulent strain in greenhouse tests.

Three sensitive strains known to have a wide spectrum of sensitivity to bacteriocin-producing strains of P. solanacearum have been employed as indicator strains to detect unknown bacteriocin-producing strains in serial dilutions of North Carolina soil samples (Chen and Echandi 1982). The technique was reported to be successful for detection and quantification of P. solanacearum, in vitro as well as in the soil samples collected from the fields. The usefulness of this technique depends on two prerequisite factors namely, reduction of other soil bacteria and fungi (hence the use of sensitive selective media) and the presence of wide spectrum indicator strains. However, this technique will not be able to detect strains that are not producing bacteriocins or producing bacteriocins to which the indicators are not sensitive.

Production of bacteriocins by Corynebacterium michiganense (now C. michiganense pv. michiganense (Smith) Jensen) has been shown in 55 of 96 strains tested (Echandi 1976). The diffuse inhibition zones, enzyme sensitivity and heat stability of bacteriocins produced by four representative strains suggest that bacteriocins corresponding to Bradley's (1967) low molecular weight group are involved. A typing scheme based on the sensitivity pattern of the indicator strains enabled typing of 90% of the strains into 10 groups. Gross and Vidaver (1979) found that the majority of bacteriocins produced by species in the genus Corynebacterium were bactericidal proteins, resistant to heat (75 - 80°C for 30 min) and sensitive to proteolytic enzymes. C. nebraskense

(Schuster et al.) Dye and Kemp, C. michiganense and C. flaccumfaciens (Hedges) Dowson and Hedges produced two bacteriocins which were differentiated on the basis of heat sensitivity, susceptibility to protease, conditions for production and spectrum of activity. A bacteriocin produced by C. michiganense strain 15-2 has been purified and characterized (Huang and Echandi 1981). The bacteriocin was found to be trypsin sensitive and heat labile (80°C for 15 min.) with bacteriophage tail-like morphology under electron microscopy. Crude bacteriocin preparations isolated from C. michiganense have been shown to be protective against bacterial canker on tomato seedlings in greenhouse experiments (Echandi 1975). Since only crude preparations were used, confirmation using the purified bacteriocin preparations would be desirable.

The best known bacteriocin associated with plant pathogenic bacteria is agrocin 84 produced by the non-pathogenic, bacteriocin producing strain 84 of Agrobacterium radiobacter pv. radiobacter Keane et al. and active against the pathogenic form of A. radiobacter pv. tumefaciens Keane et al. (New and Kerr 1972; Kerr and Htay 1974). The bacteriocin was identified as a low molecular weight, 6-N-phosphoramidate of an adenine nucleotide analogue (Roberts et al. 1977). Since there is no biologically active protein moeity in this bacteriocin, it may well represent a new type; the nucleotide bacteriocins (Kerr 1980). Bothproduction and immunity are coded for by transferable plasmids of high molecular weight, 30 x 10^6 D and 124 x 10^6 D respectively (Sciaky et al. 1977). The agrocin 84 producer also possesses another plasmid that codes for utilization of nopaline, an unusual amino acid only occurring in crown gall tissues. Kerr (1980) reported that on its own the plasmid

that confers agrocin 84 production and immunity is not conjugative but the plasmid that codes for the utilization of nopaline can promote conjugation by mobilizing the agrocin plasmid making it transferable (Ellis and Kerr 1978).

A large plasmid (Ti plasmid) was found to code for pathogenicity (Van Larebeke et al. 1975; Watson et al. 1975). The gene controlling agrocin sensitivity was also located in this plasmid (Engler et al. 1975). The ability of transconjugation between non-pathogenic, agrocin-producing and pathogenic forms of Agrobacterium has been shown in vitro (Kerr 1980). The conjugants have the possibility of having both properties, the ability to produce bacteriocin as well as being pathogenic or being both pathogenic and resistant to agrocin 84. The presence of nopaline (as a promoter) in galled tissues is a prerequisite for transconjugation.

The use of agrocin 84 in biological control programmes for crown gall has been very successful in a variety of crops in the rosaceae world-wide (Moore and Warren 1979). Biological control was possible when a ratio of at least 1:1 of non-pathogen, agrocin producer to pathogen was employed (New and Kerr 1972). Seedlings or cuttings were soaked in suspensions of A. radiobacter strain 84 before planting. In a few cases, for example in Greece, a breakdown in control has been cited probably due to the ability for plasmid transfer in the presence of already galled tissues (Kerr 1980). However in Australia little or no plasmid transfer appears to have occurred. This may be due to the fact that little or no galling has occurred, resulting in no available nopaline as a promoter. Moore (1976) reported that latent infection

resulted in 24-36% resistance to agrocin 84 in the United States. Obviously the use of healthy seedlings or cuttings inoculated with A. radiobacter strain 84 is important to ensure effective biological control. A mutant strain 84 with a defective ability for plasmid transfer would be an ideal antagonist as it would avoid the possibility of transconjugation.

In the genus Erwinia, bacteriocins from at least three species have been investigated. Bacteriocin production from E. carotovora was first shown by Hamon and Péron (1961). Later Endo et al. (1975) and Kamimiya et al. (1977) showed bacteriocin activity in the pellet fraction of culture filtrates after ultracentrifugation (105 000 x g for 60 min). Bacteriophage tail-like particles were observed in the partially purified preparations. These macromolecular bacteriocins were further purified and characterized by Itoh et al. (1978). Bacteriocin activity was recovered completely in the pellet fraction after ultracentrifugation (100 000 x g for 60 min). Carotovoricin Er production was only detected after induction with Mitomycin C. The bacteriocin was resistant to various proteolytic enzymes but was completely destroyed by heat treatment (80°C for 10 min). Echandi and Moyer (1979) showed that two strains of E. chrysanthemi also produced bacteriocins with bacteriophage tail-like particles composed of contractile sheaths and cores. Loose cores and empty sheaths were also observed. However, bacteriocin production was spontaneous and enhanced with UV light or Mitomycin C induction.

On the basis of heat and enzyme sensitivity two types of bacteriocins produced by two different strains of E. carotovora were

distinguished (Echandi 1976). Campbell and Echandi (1979) partially purified two bacteriocins which were distinguishable by agar diffusibility and ultracentrifugation. The one showing small, clear inhibition zones was found to consist of bacteriophage tail-like particles by electron microscopy. The one producing larger and diffuse inhibition zones was not studied further. A similar particle structure was confirmed by Crowley and De Boer (1980) for a bacteriocin produced by another strain of Erwinia carotovora. They noted that a low molecular weight bacteriocin was probably present together with the high molecular weight type produced by a strain in serogroup X based on agar diffusibility. The observation was not further investigated.

When semi-purified bacteriocin preparations of E. carotovora were applied prior to challenge inoculation with the pathogen, potato slices were protected from soft rot (Campbell and Echandi 1979). However protection was not observed when naturally-infested tubers were tested. No explanation for this failure was given. However, it is probable that the bacteria naturally infesting the tubers were not sensitive to the semi-purified bacteriocin preparation.

In vitro studies by Beer (1981) have shown that a non-pathogen, bacteriocin producing strain of E. herbicola inhibits the growth of E. amylovora. When living bacteria were employed, the degree of control was directly related to the bacterial concentration used. When bacteriocin preparations were used, disease incidence was greatly decreased (Beer 1981).

Itoh et al. (1980a, 1980b and 1981) have recently proposed the mode of action for carotovoricin Er. Lysis of the sensitive cells was

apparently due to degradation of the membrane phospholipid. This degradation was caused mainly by the activity of phospholipase A, an endogenous enzyme found in the sensitive cells. This enzyme seemed to be activated from a latent form by the action of the bacteriocin. However, a phospholipase A deficient mutant still retained binding to carotovoricin Er but was not lysed. This indicated that adsorption of bacteriocin to the receptor sites is an initial step leading to cell death.The presence of phospholipase A in the sensitive cells is required for lysis but how binding of bacteriocins activates lysis is unknown.

As noted above with Agrobacterium spp., production of bacteriocins may play a significant role in regulating the population dynamics of closely related species. The few in vitro studies with E. carotovora also suggest that the bacteriocin producing strains have a selective advantage over the sensitive, non-producing strains. Recently a serotyping scheme for E. carotovora has been established by De Boer et al. (1979) based on the diffusible somatic antigen. Using this scheme they noted that certain serogroups (I, III, IX, XI and XVI) are more commonly found in nature than others.

Among strains of E. carotovora subsp. atroseptica (Van Hall) Dye (Eca), those belonging to serogroup I are predominantly found associated with potato in British Columbia (Dr. R.J. Copeman, personal communication) compared to those in serogroups XVIII, XX and XXII). This project was undertaken to determine whether bacteriocin production and sensitivity is a factor in the population dynamics and distribution of strains of E. carotovora in nature. The following specific objectives were identified:

1. To determine whether bacteriocin production and sensitivity is correlated to the frequency of distribution of the various serogroups in nature.
2. To determine whether bacteriocin-producing strains are antagonistic to the sensitive strains in mixed culture studies and on potato discs.
3. To determine if more than one bacteriocin is produced and to determine whether there are differences in concentration, type and activity.

MATERIALS AND METHODS

A. Bacterial Cultures Used

All strains except SR8 of E. carotovora (see tables 1 and 2) used in these studies were originally isolated by Dr. R.J. Copeman from potatoes grown in British Columbia. Stock cultures had been maintained at 4°C for 3 to 5 years on nutrient agar slants in screw-capped tubes. Working stock cultures for routine use were maintained as turbid bacterial suspensions ($A_{660} \approx 1$) in distilled water at 4°C and renewed every 4 months. Strains were routinely streaked from the working stock cultures onto nutrient agar plates and incubated for 48 h at 26°C. Bacterial suspensions or colonies from these 48 h cultures were incubated at 26°C.

B. Screening and Detection

Bacteriocin activity was detected by the modified agar overlay technique (Crowley and De Boer 1980). Test strains were stab inoculated into nutrient agar plates. The stab inoculation technique was used in the preliminary screening and detection of the various strains in serogroups while in subsequent studies the replica plating technique was used. After 48 h incubation at 26°C, duplicate replica plates were prepared for each test indicator strain. Bacteria were killed by inverting the plates over chloroform soaked filter paper for 20 min. The colonies were scraped off the agar surface using sterile glass microscope slides. Another 20 min chloroform treatment was used to kill any remaining bacteria. Residual chloroform vapours were removed

by exposing the plates in a laminar air flow clean bench for 20 min. Suspensions of indicator strains ($A_{660} \approx 0.1$ or about 4×10^7 CFU/ml) were prepared in sterile distilled water (SDW). A 0.1 µl aliquot was added to 10 ml of molten peptone soft agar (containing 1% bacto-peptone and 0.6% agar) held at 50°C. The suspensions were mixed on a vortex mixer for about 20 s and poured over the surface of the test agar plates. These plates were then incubated at 26°C and observed after 24 h to 48 h. Zones of inhibition were rated from slight to clear on an arbitrary scale of 0 to 5. This procedure for preparation of the indicator lawns was followed in subsequent experiments unless otherwise noted.

Bacteriocin activity was detected in broth cultures by the spot assay test (Mayr-Harting et al. 1972) for strains 379, 373, 440 and 626. Test strains were grown in duplicate in 20 ml of nutrient broth in 50 ml Erlenmeyer flasks at 26°C for 48 h in a shaking water bath. Bacterial cells were removed by centrifugation (head 874 - 10 000 x g for 20 min.) at 4°C in an IEC Centrifuge Model 20-8 (from now on called low-speed centrifugation). The supernatants were filter-sterilized and three-fold dilution series were prepared in SDW. A 10 µl drop of each dilution in the series was placed in duplicate onto indicator lawns. Inhibition zones were observed after 24-48 h.

Induction experiments were done using a standard UV light source (General Electric Germicidal Tube G30T8). Strains 379, 373, 440, 625 and 626 were tested in induction experiments. Aliquots (0.1 ml) of bacterial cell suspensions ($A_{660} \approx 0.1$) of each strain were inoculated into 30 ml nutrient broth in 50 ml Erlenmeyer flasks. The flasks were

incubated on a shaking water bath for 8 h (mid-exponential growth phase). Each 30 ml was equally divided into two petri dishes and placed 26 cm below the unfiltered UV light for 20 sec. The exposed cultures were placed back into the flasks and incubated another 4 h on the shaking water bath. The suspensions received a low-speed centrifugation and the supernatants were filter sterilized. Dilution series were prepared and 10 µl aliquots were spot assayed. The experiments were duplicated and repeated twice. The plates were incubated at 26°C for 48 h before observation for inhibition zones which were recorded as positive, weak or no inhibition.

C. Partial Purification

To determine the optimal concentration of Mitomycin C (Sigma Chemical Co. St. Louis, U.S.A.) required for induction, solutions at the final concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 µg/ml were prepared in SDW. Each concentration was added at the mid-exponential growth phase to nutrient broth cultures of strains 379, 373, 440 and 626 incubated in a shaking water bath. Further incubation was done for another 6 hours. The preparations received low-speed centrifugation and the supernatants were filter sterilized. Three-fold dilution series in SDW were prepared and spot assayed onto the indicator lawns prepared as previously noted. Inhibition zones were observed after 48 h incubation and an arbitrary rating from 0 to 5 was recorded.

The bacteriocin-producing strains were cultured in 1 L of nutrient broth. Starting inoculum consisted of 1 ml of bacterial suspension ($A_{660} \approx 1$) prepared in SDW. These cultures were incubated for 8 h (to mid-exponential growth phase) on a shaker at 26°C.

Mitomycin C was added to a final concentration of 0.4 µg/ml. A further incubation of 6 h was allowed before harvest. Bacterial cells were removed by low speed centrifugation. An equal volume of saturated $(NH_4)_2SO_4$ solution was added drop-wise with stirring. The mixture was adjusted to pH 7.0 and allowed to stand overnight at 4°C. The resulting precipitates were collected by low speed centrifugation and redissolved in 10 ml 0.01 M phosphate buffer (PBS) pH 7.2 containing 0.85% NaCl. These solutions were dialysed overnight at 4°C against PBS. The dialysates were centrifuged in a Beckman LS-40, type 65 fixed angle rotor at 100 000 x g and 120 000 x g for 60 min, and at 150 000 x g for 90 min. The pellets recovered were resuspended in 5 ml 0.01M PBS. An additional low speed centrifugation removed insoluble material. The purification procedure is summarized in Figure 1. Both pellet fractions and supernatants were tested for bacteriocin activity against the sensitive indicator strains by the spot assay technique as described above.

Supernatant fractions were run on a Sephadex G-200 column (5.5 cm high and 1.5 cm diameter) previously equilibrated with 25 ml PBS. Samples (2.5 ml) were allowed to run in and PBS added. Void volumes (3.5 ml) and included volumes (8.5 ml) were collected in PBS buffer. Three-fold dilution series were prepared from the void volumes and the included volumes. Aliquots (10 µl) were spot assayed for bacteriocin activity as previously described. Activity was recorded as the last dilution at which inhibitory activity was detected on the indicator lawn.

The high speed supernatants were also fractionated on a Sephacryl S-300 column (33 cm high and 2.5 cm diameter). The column was

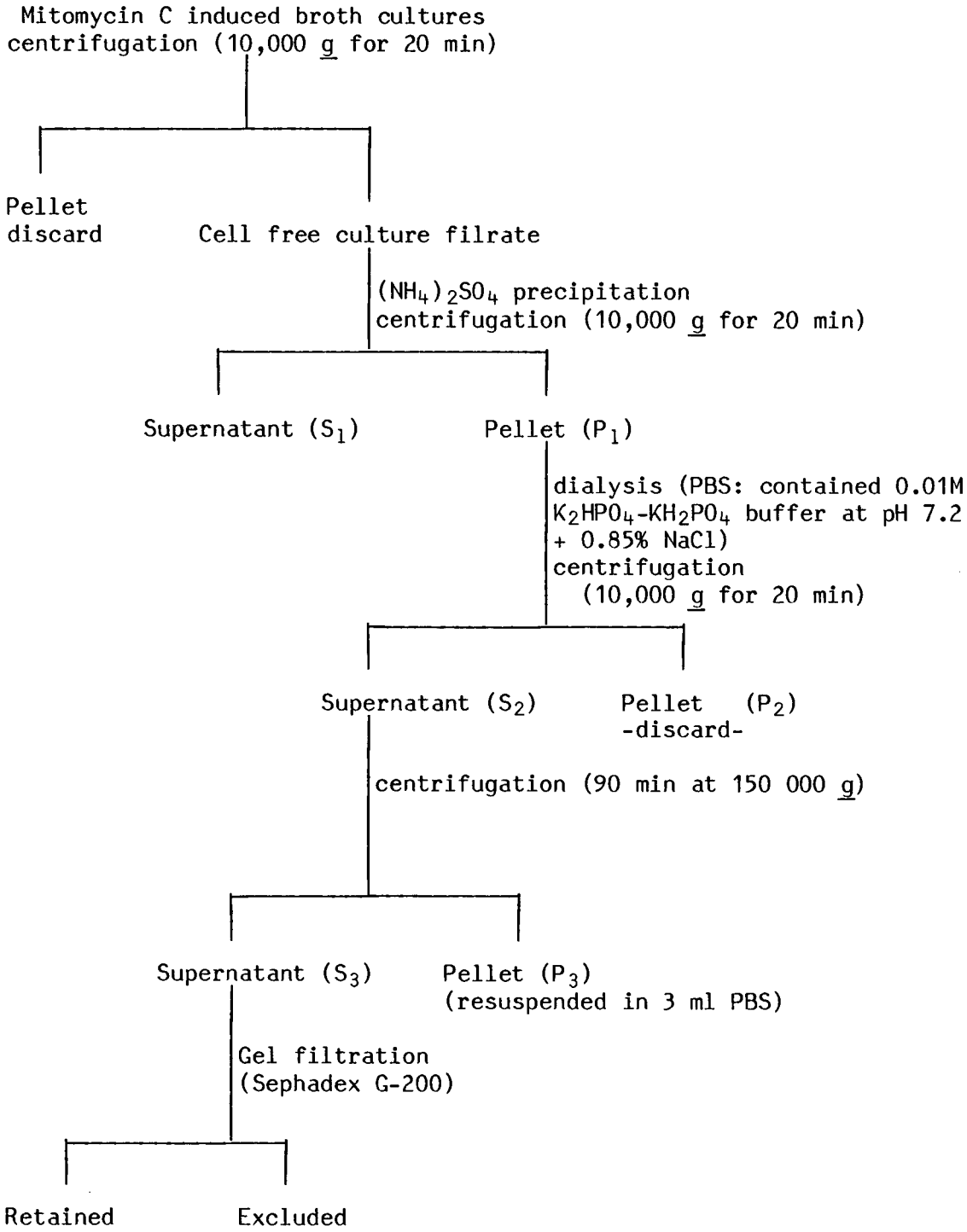

Figure 1. Flow chart for partial purification of bacteriocin from Erwinia carotovora.

equilibrated with PBS prior to use. Samples of the high speed supernatant (2 ml aliquots) were run in and 50, 2.5 ml fractions were collected at a flow rate of 2.5 ml/min. Ultraviolet absorption was monitored spectrophotometrically at 253 nm with a UV-monitor. Each fraction was tested for bacteriolytic activity against indicator strain 504 by the spot assay technique. The type and rating or diameter of the inhibition zones were recorded. A standard curve was also determined on the same column using proteins of known molecular weight, catalase (MW 2.5×10^5), apoferritin (MW 4.8×10^5), cytochrome C (MW 1.22×10^4) and phosphorylase B (MW 9.2×10^4). These proteins were run in at the concentration of 2.5 mg/ml.

Characteristics of the partially purified bacteriocin preparations were also studied. Sensitivity to trypsin (Sigma Chemical Co. St. Louis U.S.A. Type II, crude trypsin from porcine pancreas) was tested using the method of Itoh et al. (1978). However, the sample size used in the present work was 100 µl partially purified bacteriocin preparations (the resuspended pellets and supernatant fractions) from ultracentrifugation.

Stability to heat (50°C and 80°C for 10 min) was determined with 1 ml aliquots in thin-wall glass test tubes with constant shaking. The residual bacteriocin activity (after treatment with trypsin or heat) was tested by the spot assay technique using all the representative indicator strains. The presence or absence of inhibitory zones was recorded.

D. Electron microscopy

The resuspended high-speed pellets and supernatants were dropped onto copper grids coated with collodion-carbon. The grids were stained

with 2% phosphotungstic acid (20 drops) pH 7.1. Preparations were observed in a Phillips EM 300 electron microscope and photographed.

E. Dual Culture Studies

Culture conditions for these studies were maintained as previously noted. Strains 379, 373, 440 and 626 were used as representative strains of Ecc from serogroup XI and strains SR8 (I), 504 (XVIII), 530 (XX) and 496 (XXII) from the sensitive serogroups.

Bacterial suspensions ($A_{660} \approx 1$) were prepared in SDW as a starting concentration and a ten-fold dilution series (corresponding to $A_{660} \approx 1$, 0.1, 0.01, 0.001, 0.0001 and 0.00001) were prepared. The concentration of the sensitive strains was kept constant at $A_{660} \approx 0.01$ (3×10^5 CFU/ml). While the full concentration range of the producer cells was employed, the starting ratios of the producers to sensitive cells were designated as 100:1, 10:1, 1:1, 1:10, 1:100 and 1:1000. The controls consisted of the same starting ratio of a non-producer to sensitive cells as well as sensitive cells alone.

The dual cultures were vigorously mixed for about 20 s on a vortex mixer. Aliquots of 0.1 ml from the mixed cultures were inoculated into 20 ml of nutrient broth in 50 ml Erlenmeyer flasks. They were incubated in a shaking water bath. After 48 h serial dilutions were prepared and plated in duplicate onto α-MG-TZC selective medium (Phillips and Kelman 1982). The plates were incubated for 72 h at 26°C. After incubation the number of red colonies (Eca) and the number of opaque colonies (Ecc) were recorded.

In some experiments mixed cultures at the identical starting ratios were simultaneously inoculated onto potato tuber discs. The

discs were prepared from potato tubers which were surface-disinfested by immersion for 10 min in 10% Chlorox (final concentration of NaOCl 0.5%). A sterile cork borer (4mm diam.) was used to cut 3 mm-thick potato discs. The discs were placed into the wells of sterilized spot plates and each treatment replicated four times. Each disc received 0.1 ml of a mixed culture suspension. Ten fold serial dilutions of the rotted tissues were prepared in SDW and inoculated to duplicate plates of α-MG-TZC medium. Colony counts were made as noted above.

RESULTS

A. Screening and Detection

Bacteriocin production and sensitivity in E. carotovora as demonstrated by the agar overlay technique varied among strains in the eleven serogroups tested (Table 1). The one or two strains tested in each of seven biochemically E. carotovora subsp. carotovora (Ecc) serogroups (III, IV, V, IX, XI, XVI and XXI), were bacteriocin producers. The one or two strains tested in each of seven serogroups (I, III, IV, V, XVIII, XX and XXII) were bacteriocin sensitive. Three serogroups (III, IV and V) contained strains that were both bacteriocin producers and bacteriocin indicators. All E. carotovora subsp. atroseptica (Eca) serogroups (I, XVIII, XX and XXII) were found to be non-producers and bacteriocin sensitive against strains employed in this study. Representative strains in four serogroups (IX, XI, XVI and XXI) were not sensitive to bacteriocins produced by any of the eleven serogroups tested. Sensitivity to some producer strains varied between strains in the same serogroup when more than one sensitive strain was tested. With the exception of serogroup XVIII, strains in serogroups sensitive to one producer were also sensitive to at least a second producer serogroup. Serogroups III and V showed sensitivity to six and four producer serogroups respectively. At least one strain in all Eca serogroups was sensitive to the representative producing strains in serogroup XI (Ecc). Only some Eca serogroups were sensitive to the remaining producer serogroups (V, IX and XXI).

Bacteriocin production as detected by the agar overlay technique with sensitive strains in four serogroups (Eca) was not an absolute

Table 1. Sensitivity to and production of bacteriocins by strains of the predominant potato serogoups of Erwinia carotovora in British Columbia

Serogroup	Bacteriocin producing strain	Bacteriocin sensitivity of strains in serogroups														
		I		III	IV	V		IX	XI		XVI	XVIII	XX		XXI	XXII
		SR8	82	95	14	90	94	84	270	385	315	17	368	530	295	555
I	SR8,82	-[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
III	95	-	-	-	-	+	+		-	-	-	-	-	-	-	-
IV	14	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-
V	90,94	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+
IX	84	+	-	±	-	-	-	-	-	-	-	-	-	-	-	+
XI	270,385	+	+	±	-	-	-	-	-	-	-	+	-	+	-	±
XVI	315	-	-	±	-	+	+	-	-	-	-	-	-	-	-	-
XVIII	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XX	368,530	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XXI	295	-	+	+	+	+	+	-	-	-	-	-	-	-	-	±
XXII	555	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] No inhibition in all replicates, ± weak inhibition in at least one replicate, + inhibition in at least two replicates. Results of two replicates in each of two experiments.

serogroup characteristic (Table 2). Representative strains from all four serogroups were sensitive to 31/44 of the bacteriocin-producing strains in serogroup XI. These strains were designated as "typical producers". Sensitivity of strains within and among the four sensitive serogroups varied against 10 serogroup XI strains referred to as "differential producers". None of the indicator strains was sensitive to three strains classified as "non-producers". Strains in serogroup XVIII which were biochemically Ecc showed similar sensitivity patterns to strains that were biochemically Eca.

Both Mitomycin C and UV light induced bacteriocin production in nutrient broth cultures tested by the spot assay (Table 3). Noninduced broth cultures of strain 440 and the "non-producers" showed no bacteriocin activity against any of the four sensitive strains. However after induction, activity was detected in broth cultures of strain 440 by all the sensitive strains. Both non-producers showed bacteriocin activity after induction by Mitomycin C but not UV light. Only strains in serogroups XVIII and XXII showed sensitivity to bacteriocin induced in the "non-producers". Enhanced bacteriocin production by the "typical producer" strain 379 and "differential producer" strain 373 after induction by both Mitomycin C and UV light was observed as greater clearing of the inhibition zones.

The degree of clearing observed in the agar overlay technique varied among sensitive serogroups and with the strains used in any one serogroup (Table 4). "Typical producer" strains showed maximum activity (a rating of 5) against strains in serogroup I, intermediate activity against most strains in serogroup XVIII and high activity (a rating

Table 2. Sensitivity of selected strains of four Erwinia carotovora subsp. atroseptica serogroups to bacteriocins produced by strains of Erwinia carotovora subsp. carotovora serogroups XI isolated from potato in British Columbia

Producer strains in serogroup XI	Reaction of sensitive strains in serogroups																																					
	I							XVIII									XX								XXII													
	SR8	68	80	82	142	176	193	17	18	19	23	504	620	480*	481*	503*	368	369	372	375	530	531	535	537	495	496	500	541	552	555	556	557	560	562	586			
"Typical producers"																																						
41 44 350 360 361 374 377 379-384 386-390 393-400 402 405 608	+[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
270	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
"Differential producers"																																						
20	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
27	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
282	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+			
362	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
371	-	-	-	-	-	-	-	-	-	-	-	-	-	±	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
373	+	+	+	+	+	+	+	±	-	-	-	±	±	±	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	+	+	±			
376	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	-	-	+	±	+	+	-	+	+			
385	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+			
401	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	±	-	-	-	+	-	-	+	-	+	-	-	+	+			
440	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	±	+	±	+	+	-	-	-	±	±	+	-	-	+	+			
"Non-producers"																																						
351 625 626	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			

[a] + inhibition in at least two replicates, ± weak inhibition in at least one replicate, - no inhibition in all replicates. Results of two replicates in each of two experiments.

*Indicates E. carotovora subsp. caratovora strains.

Table 3. Bacteriocin activity of strains of Erwinia carotovora subsp. carotovora serogroup XI when induced with UV light and Mitomycin C as detected in the spot assay technique by sensitive strains corresponding to four serogroups of Erwinia carotovora subsp. atroseptica

Strains in serogroup XI	Bacteriocin production detected by sensitive strains in serogroups											
	I			XVIII			XX			XXII		
	SR8			504			530			496		
	Non-induced	UV light	Mitomycin C	Non-induced	UV light	Mitomycin C	Non-induced	UV light	Mitomycin C	Non-induced	UV light	Mitomycin C
Typical producer												
379	+[a]	+	+	+	+	+	+	+	+	+	+	+
Differential producers												
373	±	+	+	±	+	+	±	+	+	+	+	+
362	-	+	+	-	+	+	-	+	+	-	+	+
440	-	+	+	-	+	+	-	+	+	-	+	+
Non-producers												
625	-	-	-	-	-	+	-	-	-	-	-	+
626	-	-	-	-	-	+	-	-	-	-	-	+

[a] + inhibition in at least two replicates, ± weak inhibition in at least one replicate, - no inhibition in all replicates. Results of two replicates in each of two experiments.

Table 4. Degree of inhibition of selected strains of Erwinia carotovora subsp. atroseptica by bacteriocin - producing strains of Erwinia carotovora subsp. carotovora serogroup XI

Strains in serogroup XI	Degree of inhibition by bacteriocin sensitive strains in serogroups									
	I			XVIII			XX		XXII	
	SR8	193	142	18	504	620	530	531	495	496
Typical producers										
379	5[a]	5	5	3.5	3.5	4	5	5	3.5	4
380	5	5	5	2.5	2	2	5	4	4	4
385	5	5	5	3.5	2.5	2.5	5	4	4	4
394	5	5	5	2.5	2.5	3	4.5	4	3	4
Differential producers										
20	0	0	0	3	5	2	0	0	0	0
282	0	0	0	0	0	0	1	0	1	0
373	5	5	5	0.5	0.5	1	5	5	3	4
440	5	5	5	0	0	0	5	5	3.5	4
Non-producers										
625	0	0	0	0	0	0	0	0	0	0
626	0	0	0	0	0	0	0	0	0	0

[a]Numbers indicate degree of inhibition from slight (1) to complete (5) in the agar overlay technique. Mean rating of two replicates in each of three experiments.

of 4) against serogroup XX and XXII. "Differential producer" strains 20 and 282 showed poor or no activity against the representative sensitive strains in most of the serogroups. Strains 373 and 440 showed good activity against strains in serogroups I, XX and XXII but weak or no activity against strains in serogroup XVIII. These data suggested that the threshold quantity required by the sensitive strains to show bacteriocin sensitivity varied. The reciprocal of the dilution end-point from broth cultures containing bacteriocin activity confirmed this observation (Table 5). Strains in serogroups XVIII and XXII were more sensitive than strains in other serogroups tested to bacteriocin in noninduced broth cultures of 379. Other strains were either not sensitive or only weakly sensitive. In general three to twenty-seven fold increases in bacteriocin production were induced with Mitomycin C. Sensitivity of the four indicator strains was generally highest in bacteriocin preparations of strain 379 followed by 373, 440 and 626. Considering both induced and non-induced cultures of Ecc strains, strain 496 was the most sensitive indicator strain.

The final concentration of Mitomycin C that induced maximum bacteriocin production varied from 0.2 - 0.8 µg/ml depending upon the combination of both the producer and indicator strains (Table 6). The optimum final concentration of Mitomycin C was defined as the minimum final concentration of Mitomycin C that induced maximum bacteriocin production. These optimum values varied depending upon the combination of both the producer and indicator strains. Variability in the optimum values occurred due to differential sensitivity of the indicator strains to the bacteriocin being produced. Detectable bacteriocin activity was

Table 5. Reciprocal dilution end-point at which activity could be detected by the spot assay technique in broth culture filtrates of non-induced and induced producer strains of Erwinia carotovora subsp. carotovora serogroup XI

Strains in serogroup XI	Log_3 reciprocal dilution end-point of the bacteriolytic activity against sensitive strains in serogroups			
	I	XVIII	XX	XXII
	SR8	504	530	496
Typical producers 379				
Non-induced	1[a]	2	1	2
Induced	4	4	4	5
Differential producers 373				
Non-induced	0	0	0	2
Induced	1	4	2	4
Differential producer 440				
Non-induced	-[b]	-	-	-
Induced	1	1	2	2
Non-producer 626				
Non-induced	-	-	-	-
Induced	0	2	-	2

[a]Each value is the mean of two replicates in each of two experiments.

[b]- tested but no activity detected.

Table 6. Reciprocal dilution end-point at which activity could be detected by the spot assay technique in broth culture filtrates of non-induced and induced producer strains of Erwinia carotovora subsp. carotovora serogroup XI when induced with different concentrations of Mitomycin C

Strains			Log_3 of reciprocal dilution end-point of the bacteriolytic activity against sensitive strains with Mitomycin C (µg/ml)					
Producer	Sensitive		0	0.2	0.4	0.6	0.8	1.0
Typical								
379	SR8	(I)	1[a]	3	4	4	4	4
	504	(XVIII)	2	4	4	5	5	5
	530	(XX)	1	3	4	4	5	5
	496	(XXII)	2	3	5	6	6	6
Differential								
373	SR8	(I)	0	1	1	1	2	2
	504	(XVIII)	0	3	4	4	4	4
	530	(XX)	0	2	2	4	5	5
	496	(XXII)	2	4	4	4	4	4
440	SR8	(I)	-[b]	1	1	1	1	1
	504	(XVIII)	-	1	1	1	1	1
	530	(XX)	-	2	2	4	5	5
	496	(XXII)	-	2	2	5	5	5
Non-producer								
626	SR8	(I)	-	-	-	0	0	0
	504	(XVIII)	-	-	2	2	2	2
	530	(XX)	-	-	-	-	-	-
	496	(XXII)	-	-	2	2	2	2

[a] Each value is the mean of two replicates in each of two experiments.

[b] - tested but no activity was detected.

apparent even at the lowest final concentration of Mitomycin C tested (0.2 µg/ml) except for non-producer strain 626 where a higher final concentration (0.4 µg/ml) was required. In a few cases the lowest final concentration of Mitomycin C seemed to be all that was needed to induce maximum bacteriocin activity.

B. Partial Purification

Partial purification greatly enhanced bacteriocin activity in the "typical" and "differential producers" as detected by the spot assay test (Table 7). After $(NH_4)_2SO_4$ precipitation and ultracentrifugation the reciprocals of the dilution end-points for the resuspended pellets were higher than those from the supernatants. Strains in serogroups XVIII, XX and XXII were generally more sensitive to the resuspended pellet fractions of the "typical producer" strain 379 and "differential producer" strain 440 but this pattern of sensitivity was not clear against bacteriocin producing strain 373. Only strains in serogroups XVIII and XXII showed sensitivity to the partially purified bacteriocin of the "non-producer" strain 626. When the supernatants were fractionated on Sephadex G-200, activity was detected both in the void volumes and in the fractions retained by the columns (the included volume). The same pattern of differential sensitivity noted above was apparent in both of these fractions. Strain SR8 (serogroup I) was not sensitive to the fractions retained on the column. Only strain 504 and 496 showed differential sensitivity to the "non-producer" strain 626.

Both clear and diffuse inhibition zones were detected in the spot assay tests from the various fractions (Figure 2). The resuspended pellets always showed clear zones of inhibition of uniform diameter

Table 7. Reciprocal dilution end-point at which activity could be detected by the spot assay technique in different fractions of partially purified bacteriocin preparations

Strains and Fractions		Log_3 of reciprocal dilution end-point of the bacteridytic activity against strains in serogroups			
		I	XVIII	XX	XXII
		SR8	504	530	496
Typical producer					
379	P_3[a]	10[e]	14	15	15
	S_3[b]	3	7	6	5
	S_3 G200 ex.[c]	2	4	4	5
	S_3 G200 ret[d].	-	3	1	4
Differential producer					
373	P_3	9	7	9	9
	S_3	3	3	3	3
	S_3 G200 ex.	0	3	0	0
	S_3 G200 ret.	-	2	2	2
440	P_3	3	4	4	6
	S_3	2	4	3	4
	S_3 G200 ex.	0	2	0	0
	S_3 G200 ret.	-	2	2	2
Non-Producer					
626	P_3	-	2	-	2
	S_3	-	4	-	4
	S_3 G200 ex.	-	0	-	3
	S_3 G200 ret.	-	-	-	2

[a]Indicates pellet (3) resuspended in PBS from high speed centrifugation.

[b]Refers to supernatant (3) after high speed centrifugation.

[c]Refers to void volume (3.5 ml) collected when 2.5 ml of S_3 was run through Sephadex G-200.

[d]Refers to included volume (8.5 ml) eluted from Sephadex G-200 column.

[e]Each value is the means of two replicates in each of two experiments.

[f]- tested but no activity detected.

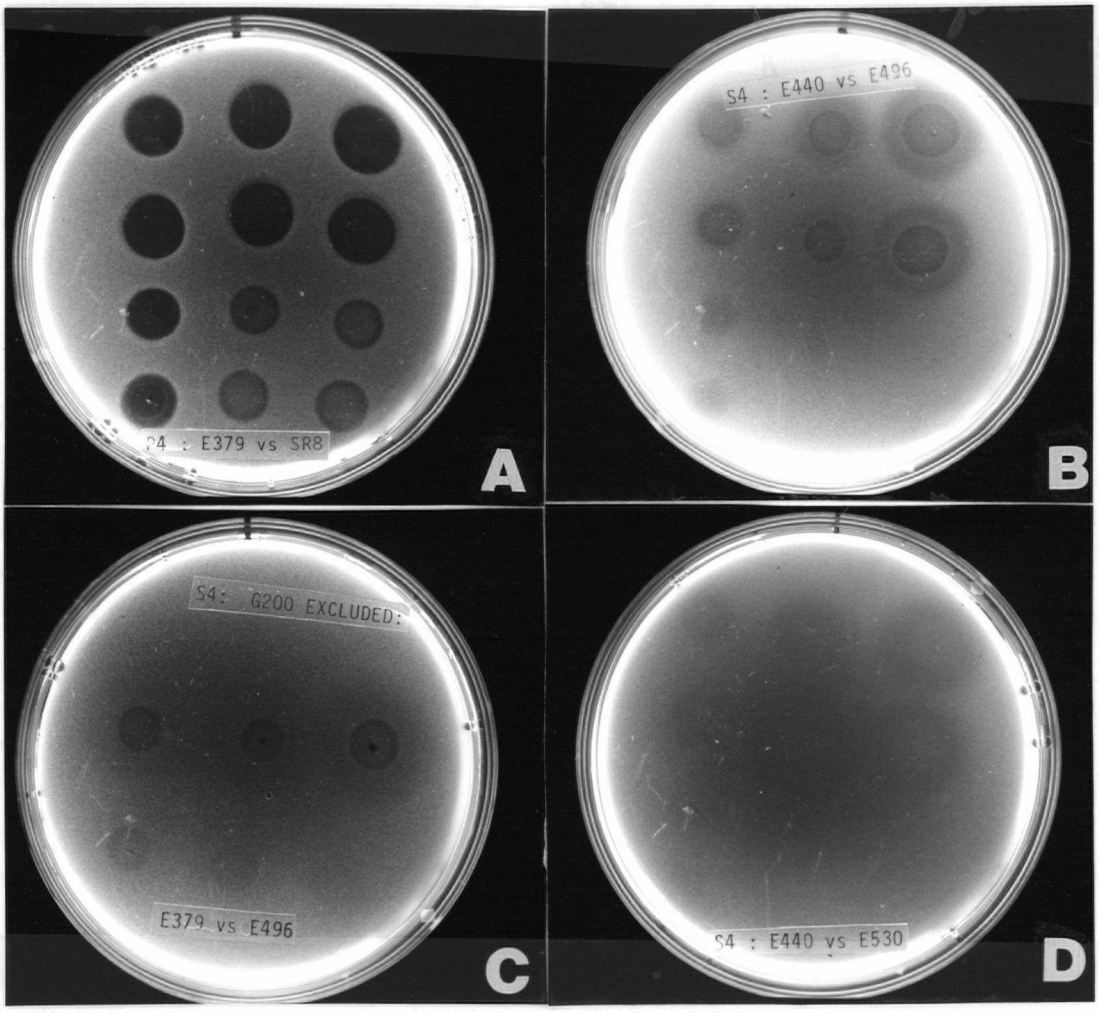

Figure 2. Bacteriocin activity against indicator strains of Erwinia carotovora subsp. atroseptica in three-fold dilution series (counter-clockwise) from Mitomycin C induced culture filtrates of Erwinia carotovora subsp. carotovora strains at different stages in purification.

A. Clear inhibition zones detected in the resuspended pellet fraction following $(NH_4)SO_4$ precipitation and ultracentrifugation for 90 min. at 150 000 x g.

B. Diffuse inhibition zones surrounding clear zones detected in the supernatant fraction following $(NH_4)_2SO_4$ precipitation and ultracentrifugation for 90 min. at 150 000 x g.

C. Diffuse inhibition zones surrounding clear zones detected in the void volume when the supernatant fractions were run on Sephadex G-200 column.

D. Diffuse inhibition zones detected in the included volume when supernatant fractions were run on a Sephadex G-200 column.

regardless of dilution (Figure 2A). This was characteristic of partially purified bacteriocin preparations against the sensitive strains in all serogroups. Clear inhibition zones were also found with all sensitive strains against the "high-speed" supernatants. But strains 504, 530 and 496 in addition had larger diffuse inhibition zones surrounding the clear zones (Figure 2B). The diameters of the clear zones remained constant until lost by dilution. The diameter of the diffuse zones decreased with dilution and were lost at lower dilutions than the clear zones. The "high-speed" supernatant fractions did not show bacteriolytic activity against the producing strains. Mitomycin C control (at the final concentration of 0.4 mg/ml) gave only small (2mm) and weak inhibition zones. These results suggested that two bacteriocins were present at different concentrations or that the sensitivity of the indicator strains to the two types of bacteriocins varied.

When the partially-purified supernatants from the typical and differential producers were fractionated on Sephadex G-200, both clear and diffuse zones were associated with the void volumes (Figure 2C). All the indicator strains showed a similar sensitivity. In contrast only diffuse zones were associated with the retained fractions (Figure 2D) detected by strains 504, 530 and 496.

Further separation of the high speed supernatant on Sephacryl S-300 showed activity against strain 504 (serogroup XVIII) in two series of fractions (Figure 3). Fractions 17-24 showed only clear inhibition zones whereas fractions 33-46 showed only diffuse zones. Serial dilutions of fractions 33-46 resulted in an activity spectrum suggesting

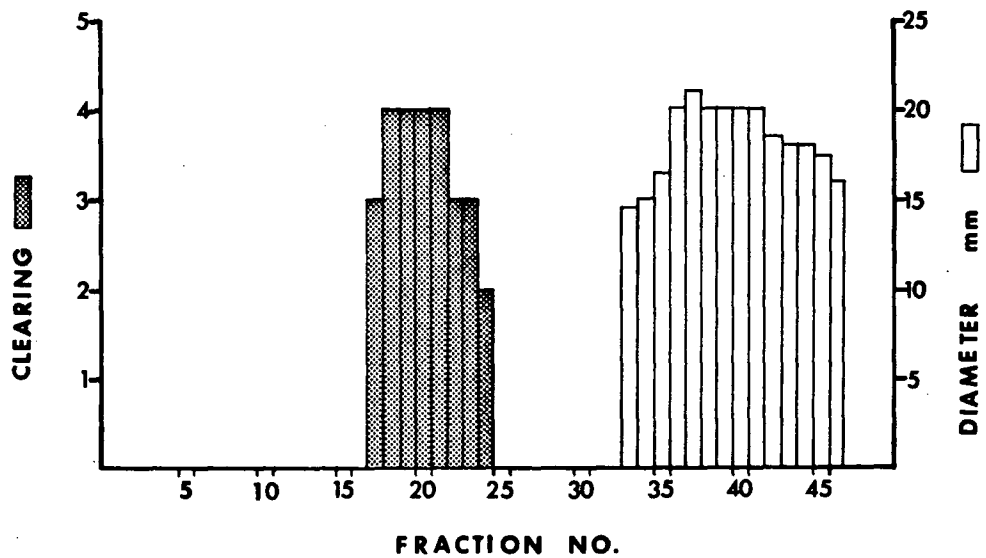

Figure 3. Bacteriocin activity (detected by indicator strain 504) in the partially purified "high speed" supernatant (strain 379) after fractionation on a Sephacryl S-300 column.

a maximum of two peaks with only weak evidence for the second peak (Figure 4). The estimated molecular weights calculated from the peaks of the inhibitory activity in fractions 17-24 was about 224 500 D, in fractions 33-39 was about 29 500 D and in fractions 40-46 was about 17 500 D (Figure 5).

Resuspended high speed pellet preparations when visualized under the electron microscope contained bacteriophage tail-like particles (Figure 6A and 6B). The dimensions of the particles were about 139 x 20 nm. Most particles from strain 379 preparations showed contracted sheaths situated at various position along the inner cores. Some particles were visualized as empty sheaths without the inner cores, others just as the inner cores without the sheaths. The empty sheaths were more frequently observed in preparations from strain 440. However, no bacteriophage tail-like particles or components were observed in the supernatant fractions.

All four fractions which showed bacteriocin activity or "bacteriocin-like" activity were heat labile when subjected to 80°C for 10 min. However, the four bacteriolytic fractions were not sensitive to trypsin.

C. Antagonism in Dual Cultures

In vitro antagonism was demonstrated in dual broth cultures of the producer and sensitive strains (Table 8). The growth of sensitive strains in serogroups XVIII, XX and XXII was not detected even at the starting ratio of approximately one typical producer cell to 1000 sensitive cells. Growth of strain SR8 (serogroup I) was prevented at the starting ratio of one producer to 10 sensitive cells. The activity

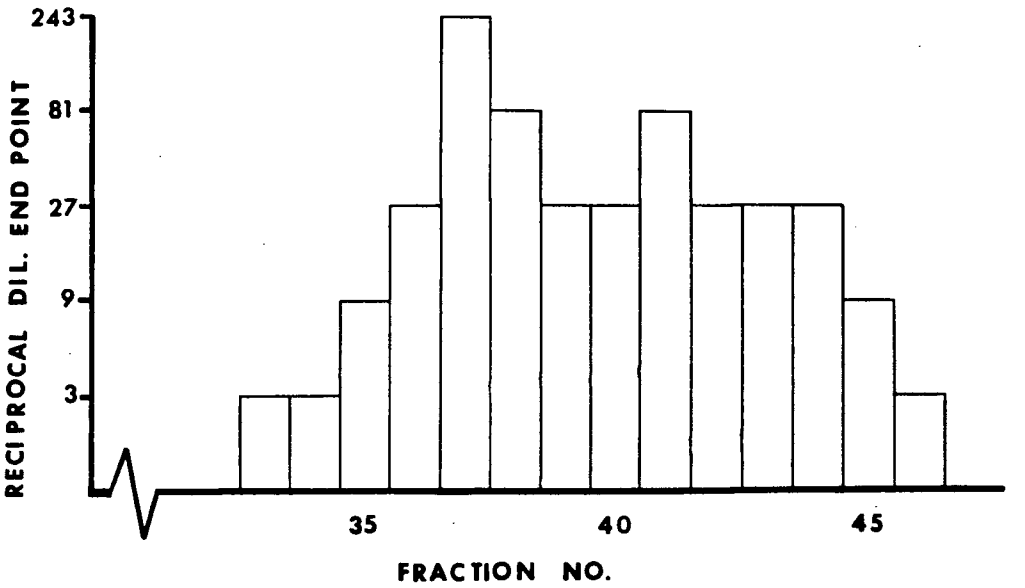

Figure 4. Relative bacteriolytic activity (detected by indicator strain 504) in fractions 33 to 46 obtained when the partially purified high speed supernatant from producer strain 379 was run on a Sephacryl S-300 column.

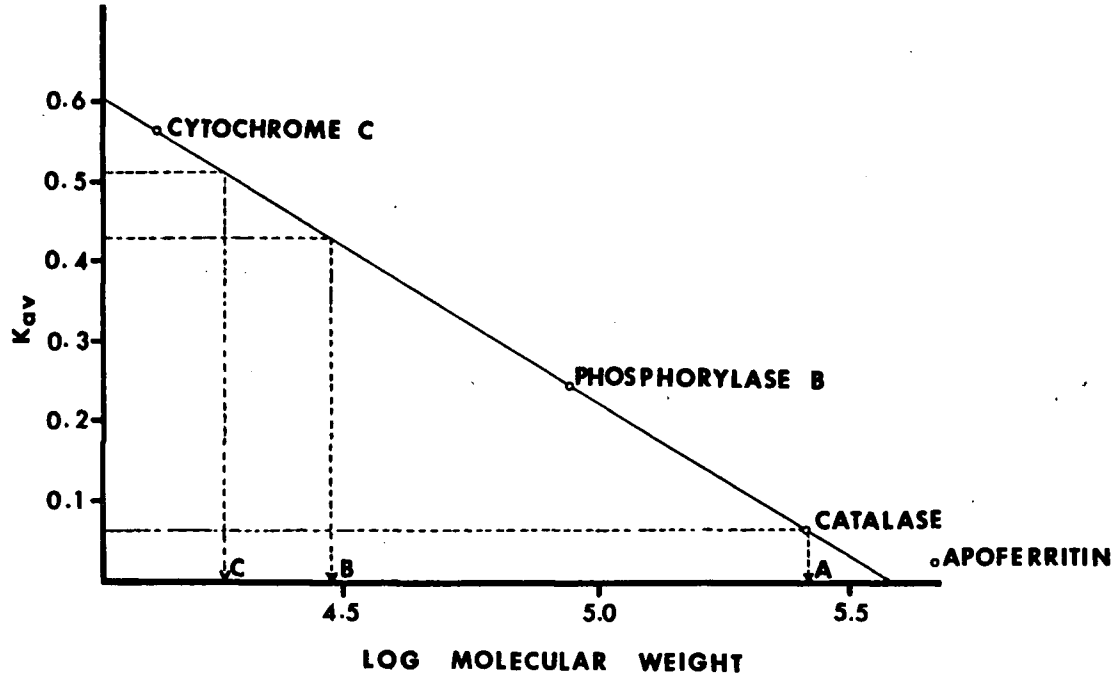

Figure 5. Standard curve of Kav Vs log molecular weight plotted using protein of known molecular weights (apoferritin, catalase, phosphorylase B and cytochrome C) determined from gel filtration (Sephacryl S-300). (A) indicates Kav Vs log molecular weight of the bacteriocin-like component at fraction #19, at fraction #37 (B) and at fraction #41 (C).

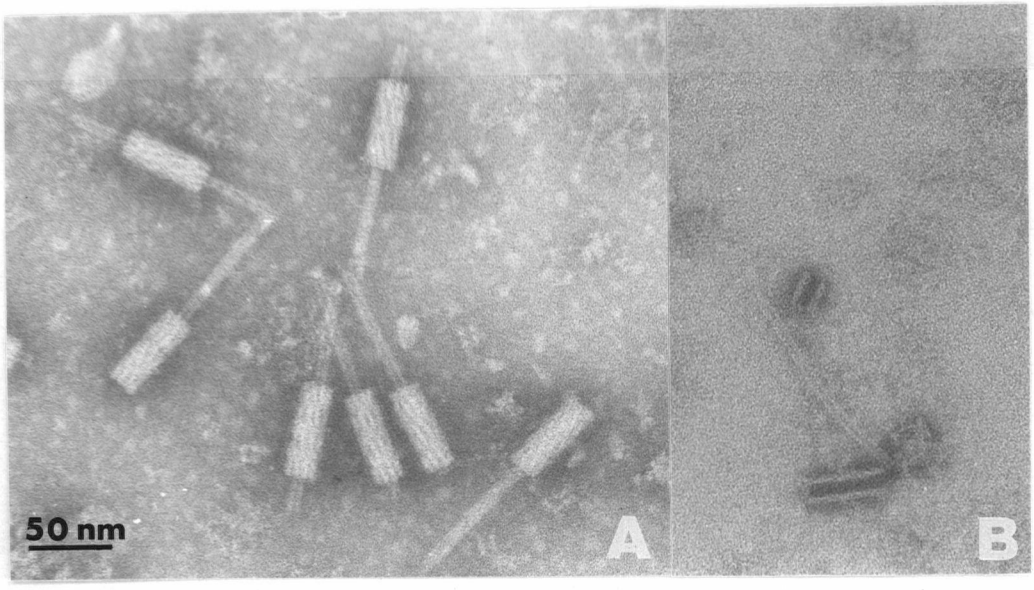

Figure 6. Electron micrograph of resuspended high speed pellet fraction from E. carotovora strains 379 (A) and 440 (B).

Table 8. Detection of sensitive strains of Erwinia carotovora subsp. atroseptica after 48 h incubation in dual broth cultures with different starting ratios of Erwinia carotovora subsp. carotovora serogroup XI

Strains in serogroup XI	Sensitive strains and serogroups		Detection at starting ratios of producer to sensitive strains 100:1	10:1	1:1	1:10	1:100	1:1000
"Typical producer"								
379	SR8	(I)	-[a]	-	-	-	+[b]	+
	504	(XVIII)	-	-	-	-	-	-
	530	(XX)	-	-	-	-	-	-
	496	(XXII)	-	-	-	-	-	-
"Differential producers"								
373	SR8	(I)	-	-	-	-	-	+
	504	(XVIII)	-	-	+	+	+	+
	530	(XX)	-	-	-	-	-	-
	496	(XXII)	-	-	-	-	-	+
440	SR8	(I)	-	+	+	+	+	+
	504	(XVIII)	+	+	+	+	+	+
	530	(XX)	-	-	+	+	+	+
	496	(XXII)	-	-	-	+	+	+
"Non-producer"								
626	SR8	(I)	+	+	+	+	+	+
	504	(XVIII)	+	+	+	+	+	+
	530	(XX)	+	+	+	+	+	+
	496	(XXII)	+	+	+	+	+	+

[a] - no detectable growth of Erwinia carotovora subsp. atroseptica on α-MG-TZC medium after 72 h. Results of two replicates in each of two experiments.

[b] + growth of Erwinia carotovora subsp. atroseptica detected on α-MG-TZ medium after 72 h. Results of at least two replicates in each of two experiments.

spectrum of *in vitro* antagonism for the "differential producers" varied between strains. "Differential producer" strain 373 was generally more antagonistic than strain 440 and both displayed a completely different activity spectrum against the sensitive strains. Growth of strains SR8, 504 and 496 was not detected in the mixed cultures with "differential producer" strain 373 at the low starting ratios (1:100 or 1:1000) of producer to sensitive cells. A higher starting ratio of producer to sensitive cells (1:1 or higher) was needed to eliminate the growth of the latter in mixed cultures of strain 373 against 504 and strain 440 against SR8, 530 and 496. "Differential producer" strain 440 was not an effective antagonist against strain 504. By contrast the "non-producer" permitted the growth of all the sensitive strains even at the highest starting ratio (100:1) of the former to the latter. Because of the apparently longer lag phase of *Eca* compared to *Ecc* (Figure 7) the ratios after 48 h in mixed cultures would not be less than those at starting time.

When mixed cultures were inoculated onto potato discs, a pattern of antagonism similar to that in dual broth cultures was observed (Table 9). Depending upon the producer strains and the sensitive strains employed, the growth of the sensitive strain was completely inhibited by a starting ratio of one "typical producer" to 100 or 1000 sensitive cells. By contrast the non-producing strain 626 at a starting ratio of 100 to one did not prevent growth and detection of the sensitive indicator strain.

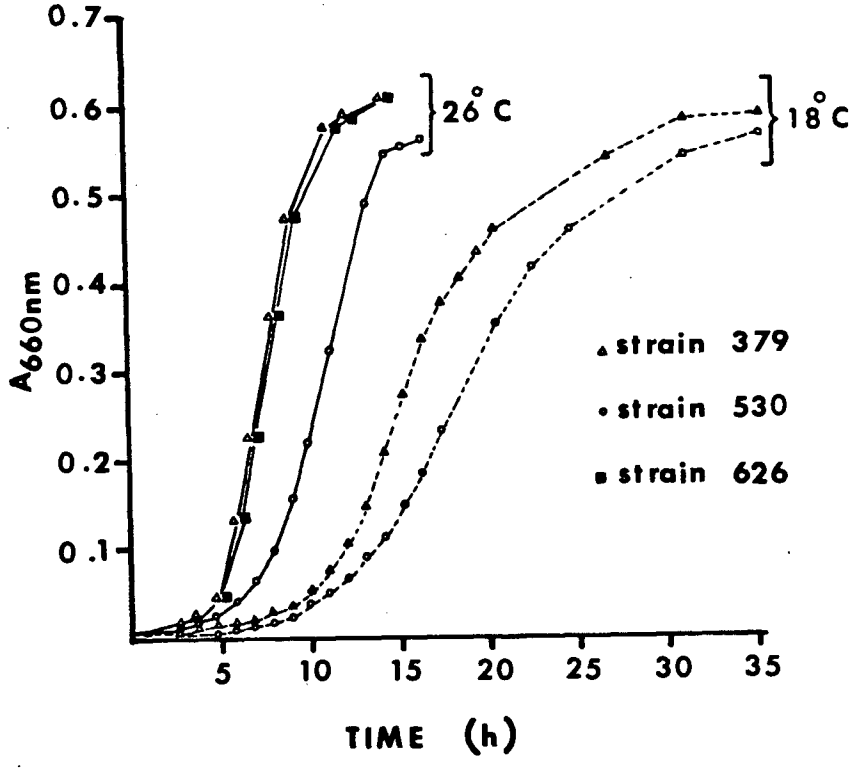

Figure 7. Growth curves for strains 379 and 626 Erwinia carotovora subsp. carotovora) and strain 530 (Erwinia carotovora subsp. atroseptica) in nutrient broth at two different temperatures.

Table 9. Detection of sensitive strains of Erwinia carotovora subsp. atroseptica in potato discs 48 h after inoculation with mixed cultures containing different starting ratios of Erwinia carotovora subsp. carotovora serogroup XI

Producer strain	Non-producer strains	Sensitive strain	Detection at starting ratios of producer to sensitive strains					
			100:1	10:1	1:1	1:10	1:100	1:1000
379		530	-[a]	-	-	-	-	-
	20,626	530	+[b]	+	+	+	+	+
385		496	-	-	-	-	-	+
	371	496	+	+	+	+	+	+

[a] - no detectable growth of Erwinia carotovora subsp. atroseptica on α-MG-TZC medium after 72 h. Two replicates in each of two separate experiment.

[b] + growth of Erwinia carotovora subsp. atroseptica on α-MG-TZC medium after 72 h. Results of at least two replicates in each of two separate experiments.

DISCUSSION

A. Screening and Detection

Based on the one or two representative strains from each serogroup used in the preliminary screening and detection experiments, bacteriocin production varied among serogroups (Table 1). Only strains which were biochemically Ecc produced bacteriocins. Campbell and Echandi (1979) reported that both Eca and Ecc were able to produce bacteriocins spontaneously in natural media which suggests that bacteriocin production is not a subspecies characteristic. However, their observation was not confirmed in the present work. Bacteriocin production by strains in serogroup V, IX and XI was observed which confirmed the report of Crowley and De Boer (1980). In addition a representative strain in serogroup XXI, not tested in the previous report, was also shown to be a bacteriocin producer.

All serogroups containing Eca strains and some serogroups containing Ecc strains were sensitive to one or more bacteriocins of the producing strains (Table 1). Eca strains in two new serogroups tested in this study were also observed to be sensitive, non-producers. Sensitivity of strains within serogroups was sometimes found to be variable in those instances where two representative strains in each serogroup were tested. Some strains had a wide sensitivity spectra. For example, most strains in serogroups I, III, V and XXII were sensitive to bacteriocins produced by strains in several different serogroups. By contrast, some strains had a narrow sensitivity spectra. For example representative strains in serogroups V, XVIII and IV were sensitive to only bacteriocins produced by strains in serogroups III, XI

and XXI respectively. In addition one or two representative strains in each of four serogroups were sensitive to the bacteriocins produced by strains in serogroups XI and XXI.

Detailed investigation of several strains in one Ecc and four Eca serogroups suggested that bacteriocin production and sensitivity in these strains at least, was a serogroup characteristic (Table 2). The fact that serogroup XVIII strains which were either Eca or Ecc reacted similarly further supported the conclusion that bacteriocin sensitivity is a serogroup characteristic rather than a subspecies characteristic.

There is no conclusive evidence that the ability to produce bacteriocins plays a major role in regulating the occurence of the predominant serogroups in nature. Several lines of evidence do not support this hypothesis. The most common serogroup (I) is bacteriocin sensitive and a non-producer. Another common serogroup (III) is also bacteriocin sensitive but produces a bacteriocin to which only a rarely-found serogroup is sensitive. In addition strains in three rarely-isolated serogroups (XXI, IV and V) were bacteriocin producers. However, some evidence supporting the hypothesis was also obtained. Strains in three common serogroups (IX, XI and XVI) were bacteriocin producers and were non-sensitive. Also strains in the rarely-isolated serogroups (XVIII and XX and XXII) were all sensitive non-producers. It is possible that bacteriocins may play a role in the distribution of only certain serogroups but do not represent a general mechanism in all.

Some strains previously reported by Crowley and De Boer (1980) to be non-producers did produce bacteriocins in this study. Strain 95 (identical to strain 195 in the previous study) was reported to be a

non-producer, sensitive to producer strains in serogroups V, IX, XI and XII. The present work confirmed the sensitivity towards the first three isolates (the fourth was not tested). However strain 95 was found to be a producer against serogroup V strains. Similarly, strains 14 (identical to strain 190 in the previous study) and 315 (identical to strain 94 in the previous study) in serogroup IV and XVI respectively were reported as being neither sensitive nor producer strains but both were found to be antagonistic against strains in serogroup III and V (Table 1). Strains 90 and 94 in serogroup V were not sensitive to producer strain 385 (serogroup XI) or producer strain 84 (serogroup IX). Such a result is not unexpected with strain 385 since Crowley and De Boer (1980) found that only 14% of the strains in serogroup V were sensitive to this strain. However, the observed insensitivity to strain 84 where Crowley and De Boer (1980) had previously found 100% of the strains tested in serogroup V to be sensitive was not expected. Since strain 84 did produce bacteriocin which was detected by indicator strains SR8, 95 and 555, the reason for the insensitivity of strains in serogroup V is not known. Similarly, strain SR8 (serogroup I) was sensitive to strain 84 (identical to strain 63 in the previous study) but none of the 13 strains in serogroup I tested by Crowley and De Boer (1980) was sensitive to it.

The discrepancies with the previous report may be due to the possibility that some of the strains lost the plasmids which confer bacteriocin production. The plasmids determining a variety of properties in many different bacteria are generally stable (Elwell and

Shipley 1980). However, plasmids are transferable (Ellis and Kerr 1978; Broda 1979) and are lost at higher rates than normal mutation frequency (Broda 1979).

Temperature of incubation may also have some effect on detection. In one instance when cultures were incubated accidently at 32°C, poor or no inhibition zones was observed. By contrast incubation at 26°C gave good inhibition zones. Crowley and De Boer (1980) used 28°C. Whether reduced production due to the 2°C temperature difference was another reason for the discrepancies remains to be determined. However, Vidaver et al. (1972) have reported that the optimum growth temperature (28 to 32°C) of some Pseudomonas spp. was higher than the optimum temperature for bacteriocin production (20 to 24°C).

A thorough investigation of the interaction between representative strains in the four Eca serogroups against a natural population of strains in serogroup XI indicated that both bacteriocin sensitivity and spontaneous production were not absolute serogroup characteristics. Variability in the sensitivity of strains in serogroup I and XVIII was not reported by Crowley and De Boer (1980) but was common in the other serogroups studied by them. The fact that the present study showed variability in both plus the two additional Eca serogroups was probably because 44 producing strains in serogroup XI were tested instead of just one strain as used by them. This variability was used to group the population of serogroup XI strains into "typical", "differential" and "non-producer" strains. Since bacteriocin production could be enhanced in "differential producers" by both UV and Mitomycin C and induced in "non-producer" strains by

Mitomycin C, the ability to produce bacteriocins may well be a serogroup characteristic. Apparently, the expression of this potential seems to have been lost in some strains.

Demonstration of bacteriocin production by the same strain varied depending upon the techniques used (Tables 4 an 5). For the purpose of detection alone, the agar overlay technique was more sensitive than the spot assay from the culture filtrate of the uninduced culture preparations. There are several reasons for this observation. In the agar overlay technique growth of the producer cells is concentrated in a small area maximizing the concentration of bacteriocin. Bacteriocin production in broth cultures by contrast was distributed in a large volume that diluted the actual amount released by the producers. However, the potential for quantifying the concentration of bacteriocins produced by determining the dilution end-point, makes the spot assay technique especially useful with high-titred preparations recovered after induction or purification. The spot assay method is also highly reproducible under standardised experimental conditions (Mayr-Harting et al. 1972).

The arbitrary numerical ratings given in the agar overlay technique for the degree of inhibition did not correspond to the titre of bacteriocin produced in broth cultures as shown by the dilution end-points in the spot assay. Bacteriocin produced by strain 379 tested against 504 gave a 3.5 rating whereas against strain SR8, it gave a 5 rating. Assuming that the same relative production occurs in liquid and solid media the 3.5 rating had in fact three fold higher bacteriocin titre than the 5 rating in uninduced culture. This result showed that

the degree of clearing in the agar overlay technique was not a good parameter in quantifying bacteriocin production. The spot assay technique was a more accurate quantitative measurement of the bacteriocin titre. However, induction was necessary since only small amounts were produced in uninduced cultures (Table 5).

Although bacteriocin production was induced by both UV and Mitomycin C, the latter was preferred for several reasons. Only Mitomycin C was able to induce the non-producer strains to produce bacteriocin (Table 3). Optimum UV light exposure (as determined by Crowley and De Boer 1980) did not result in detectable bacteriocin production in the non-producer strain. Mitomycin C was more convenient to use since it involved the addition of the chemical to exponentially growing cultures. It also provided a more uniform contact with the individual cells. By contrast, only cells on the surface have greater access to the UV light exposure. Echandi and Moyer (1979) found that the duration of UV exposure was crucial because greater or less than 30 s or 60 s (depending on strains) actually reduced the bacteriocin titre. Haag and Vidaver (1974) working with Syringacin 4-A production by Pseudomonas syringae strain 4-A also found that Mitomycin C induced 100 fold more bacteriocin activity compared to induction using UV light.

Different optimum final concentrations of Mitomycin C (0.2 - 0.8 µg/ml) were found to induce maximum bacteriocin production depending on the combination of producer and sensitive strains (Table 6). The optimum final concentration of Mitomycin C in other studies also varied, 0.8 - 1.0 µg/ml for P. syringae (Haag and Vidaver 1974) and 1 µg/ml for Corynebacterium michiganense (Echandi 1976). When higher final

concentrations of Mitomycin C (1.0 - 2.5 µg/ml) were used, the bacteriocin production by E. chrysanthemi levelled off (Echandi and Moyer 1979). Similar patterns were observed in this study.

B. Partial Purification

Partial purification and concentration of the bacteriocins produced by strains in serogroup XI resulted in bacteriolytic activity being detected in both the supernatant and resuspended pellet fractions following ultracentrifugation. Activity was not detected in the pellet fractions resulting from a 1 h centrifugation at 100 000 x g and 120 000 x g as reported by Itoh et al. (1978) and Crowley and De Boer (1980). However, high bacteriolytic activity was obtained in the resuspended pellet fractions after a 90 min ultracentrifugation at 150 000 x g and typical bacteriophage tail-like particles (139 x 20 mm) were observed by electron microscopy. The dimensions of the particles estimated from the elecron micrographs in the report by Itoh et al. (1978) and Crowley and De Boer (1980) were 176 x 20 mm and 156 x 20 mm respectively. The fact that the particle size calculated in this work was smaller might explain why activity was not detected at the lower centrifugation speeds.

Production of rapidly diffusing small molecular weight bacteriocins as well as the macromolecular type by a single strain of E. carotovora was previously noted by Crowley and De Boer (1980). Production of either one type or the other by different strains of E. carotovora has also been reported by Campbell and Echandi (1979). However, this is the first report, clearly establishing the production of both types of bacteriocins in a single strain of E. carotovora. The bacteriolytic activity in the supernatant was resolved into components

which gave distinctly different reactions in the spot assay. Both non-diffusing clear zones of inhibition and spreading diffuse zones of inhibition were observed. A high molecular weight component which was excluded from a Sephadex G-200 column and eluted off first on a Sephacryl S-300 column was responsible for the clear inhibition zones. The estimated molecular weight (224 500 D) corresponded with the non-diffusibility observed. The second peak of activity eluted from Sephacryl S-300 gave only diffuse inhibition zones. Reciprocal dilution end-points of the bacteriocin activity of each fraction suggested that there were two peaks which were not well separated by this column. The dilution end-points between pellet fractions and the supernatants, while not strictly comparable due to differences in volume, would suggest that the macromolecular bacteriophage tail-like particles are responsible for most of the bacteriolytic activity.

The high-speed supernatant fractions were classified as "bacteriocin-like" compounds because they were active against closely related strains but not active against the producing strains. The bacteriolytic activity was also not due to residual Mitomycin C. The diffuse zones were detected only in the spot assays from induced culture preparations after partial purification and concentration. This suggests that the small molecular weight "bacteriocin-like" compounds are either produced in low concentration and are not detected by the sensitive indicator strains or that they are perhaps breakdown products of the macromolecular bacteriocins. However, Itoh et al. (1978) reported that denatured carotovoricin Er is easily digested by trypsin whereas "bacteriocin-like" compounds described in the present study were trypsin resistant.

All four bacteriolytic fractions (the macromolecular phage tail-like particles plus three "bacteriocin-like" components were produced in different concentrations by all types of serogroup XI (Table 6). Production by the "typical producer" was highest and the "non-producer"the lowest. A larger quantity of the "bacteriocin-like" components is needed to confirm the insensitivity of the indicator strains SR8 and 496 to the bacteriocins induced in the "non-producers".

C. Dual Culture Studies

Dual culture studies clearly indicated that when producer and indicator strains were growing together, inhibition of the sensitive strains occurred. The fact that starting ratios of one producer to 100 or 1000 sensitive cells resulted in inhibition by the "typical producer" strain and a ratio of 100 "non-producer" to one sensitive cell did not prevent detection of the latter eliminates nutrient competition as the mechanism of inhibition. Since the producers and the "non-producers" have similar growth rates (Figure 2), the antagonistic behaviour observed with the producers probably is due to the production of bacteriocins. While bacteriocin activity itself was not monitored in either the dual cultures or potato disc experiments, bacteriocin production in similar broth cultures have been demonstrated in other experiments (Table 5 and 6).

The demonstrated ability of the "typical producer" strains (serogroup XI) to antagonise a large number of sensitive cells would give the producing strains a selective advantage in nature and be ideally suited for use in biological control work. In the A. radiobacter - A. tumefaciens system at least a one to one ratio of

non-pathogenic bacteriocin producer to pathogenic cells was needed to result in complete protection from crown gall. Since E. carotovora is pathogenic to potato the idea of using virulent, bacteriocin-producing strains to control disease is not attractive. A possible alternative would be to use purified bacteriocin preparations. Partially purified bacteriocin from E. carotovora applied prior to inoculation with the pathogen has been reported by Campbell and Echandi (1979) to control soft rot on potato slices in vitro. The same preparation failed to control soft rot of whole naturally-infested potato tubers. Purified Syringacin 4-A, a bacteriocin produced by Pseudomonas syringae strain 4-A, has been used experimentally to control P. phaseolicola strain BE and for seed treatment against P. glycinea (Vidaver 1976).

Bacteriocins have the potential of being an effective biological control against the sensitive strains in the long run since they are more acceptable environmentally and more selective against the target organisms than very broad-spectrum chemicals and antibiotics. There is also a possibility of expanding the ability to produce bacteriocins in non-producer strains. In Agrobacterium the bacteriocinogenic factor has been shown to be coded by plasmid genes (Sciaky et al. 1978; Merlo and Nester 1977). This plasmid is also transferable in the presence of nopaline which behaves as a promoter (Ellis and Kerr 1978). If similar plasmids exist in the E. carotovora system, the potential exists for introducing the genes determining production of various bacteriocins into a single strain. Through genetic engineering, a non-pathogenic ideal antagonist producing bacteriocins with a wide activity spectrum may be created. However, the deletion of other characteristics enabling survival of the bacteria in nature should not be ignored.

SUMMARY

1. Bacteriocin production by strains in serogroup XI (Ecc) and bacteriocin sensitivity of strains in serogroups I, XVIII, XX and XXII (Eca) were generally a serogroup characteristic.
2. All Ecc strains tested were bacteriocin producers at least after mitomycin C induction.
3. Some Ecc strains were both bacteriocin sensitive as well as being bacteriocin producers.
4. All Eca strains tested regardless of serogroup were bacteriocin sensitive and non producers.
5. Production of and sensitivity to bacteriocins were not correlated with the frequency of distribution of strains in the most commonly isolated serogroups (I and III) in British Columbia.
7. Bacteriocin-producing strains were antagonistic to sensitive strains in mixed cultures in vitro and on potato discs.
8. Bacteriophage tail-like particles present in the pellet following $(NH_4)_2SO_4$ precipitation and ultracentrifugation were present in the highest concentration and are responsible for bacteriolytic activity in vitro.
9. "Bacteriocin-like" activity in the high speed supernatants was resolved by gel filtration into three components with estimated molecular weights of 17 700, 29 500 and 224 500 D.

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