BACTERIOCIN PRODUCTION IN

ERWINIA CAROTOVORA SUBSPECIES *CAROTOVORA* STRAIN 379

By

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ABSTRACT

Erwinia carotovora subsp. *carotovora* strain 379
(*Ecc* 379) produced a particulate bacteriocin called
carotovoricin-379 which resembled a bacteriophage
tail. Carotovoricin-379, producing both clear and
diffuse zones of growth inhibition, was active
against several other *Erwinia carotovora* strains.
It was detected in the filter-sterilized
supernatant of *Ecc* 379 under standard cultural
conditions. Its concentrations in the supernatant
fraction of cultured *Ecc* 379 were increased by
induction with mitomycin C (0.2 ug/ml). Induction
was followed by cell lysis, which was reflected by
a sharp reduction in culture turbidity. Growth of
Ecc 379 at 37 C with or without mitomycin C
resulted in the loss of particulate carotovoricin-
379 production. Under these conditions, a low
molecular weight, highly diffusible bacteriocin
component was detected which produced large diffuse
zones of inhibition with three of the four standard
Erwinia carotovora indicator strains used. Cell
lysis following induction and a wild-type
resistance to erythromycin and chloramphenicol were

also temperature sensitive.

Carotovoricin-379 production was investigated by isolating intermediates at several times after induction. These intermediates were analysed by electron microscopy (EM) and Sephacryl S-300 column chromatography. EM showed a subunit-like arrangement of bacteriocin components around a central core. Carotovoricin-379 particles were initiated as fimbrae-like projections which matured by the gradual external addition of bacteriocin components. This maturation process was accompanied by a general increase in molecular weight, bioactivity and ultrastructural appearance of particulate carotovoricin-379. By using a modified negative staining protocol, cell projections which resembled carotovoricin-379 intermediates were seen physically attached to intact producing cells. On the basis of these results a hypothetical model for carotovoricin-379 production was outlined.

Genetic analysis of a temperature sensitive particulate bacteriocin production in *Erwinia carotovora* subsp. *carotovora* strain 379 (*Ecc* 379) was performed with and without mobilization vector

R68.45. *E. coli* transconjugants which both produced
particulate carotovoricin-379 and degraded crystal
violet pectate medium (CVP) were produced only when
R68.45 was used. In addition, the transfer
frequencies obtained were indicative of
chromosomally derived determinants. Erythromycin-
and chloramphenicol-resistant transconjugants were
obtained regardless of R68.45 mediation. DNA
analysis by agarose gel electrophoresis showed that
Ecc contained a resident megaplasmid which was
self-transmissible. This plasmid coded for
erythromycin and chloramphenicol resistance along
with the production of the low molecular weight
carotovoricin-379 component similar to that
produced by heat treated *Ecc* 379. Analysis of
transconjugants by electron microscopy showed a
protuberance of surface "blebs" or vesicles. In
addition, alkaline phosphatase (a periplasmic
enzyme) was detected in supernatants of
transconjugants. These facts implied that the
megaplasmid may also be involved in the release of
many exo-proteins of *Erwinia*.

Polyclonal rabbit antiserum was developed
against particulate carotovoricin-379. This

antiserum was tested against culture supernatants from wild-type *Erwinia* and several *Erwinia* x *E. coli* transconjugants using radial immunodiffusion (RID), immunosensitive electron microscopy (ISEM) and Western blotting. The polyclonal antiserum against particulate carotovoricin-379 reacted with supernatant fractions from all transconjugants. CVP^+ transconjugants consistently showed a wider range of reactive bacteriocin components than CVP^- transconjugants. A low molecular weight partially active carotovoricin-379 component was detected in CVP^- transconjugants by antiserum raised against particulate carotovoricin. This suggested a relatedness between the two major bacteriocin components of carotovoricin-379.

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GENERAL INTRODUCTION

Erwinia carotovora subsp. *carotovora* (Jones)
Bergey *et al.* is responsible for soft-rot of a
variety of economically important agricultural
crops. These bacteria are opportunistic pathogens
and survive as saprophytes. They do not attack
healthy plant material but are able to invade
wounded, weakened or old plant tissues. Within
infected material, the bacteria multiply in the
intercellular spaces and secrete a variety of
enzymes which degrade the middle lamella and cell
wall components. This results in a softening and
separation of plant cells followed by water loss
and eventual plant cell death. Because these
potential pathogens may be found in soil as natural
microflora, one possible means of control involves
the selective inhibition of these organisms by non-
pathogenic rhizosphere inhabitants. The simplest
conceivable means of this type of bio-control would
be the production of an inhibitory substance (ie. a
bacteriocin) by some non-pathogenic organism which
was specifically active against *Erwinia carotovora*.

An alternative approach might involve the

transfer of genetic information for bacteriocin
production to a non-pathogenic rhizosphere
inhabitant. This latter approach requires a
genetic segregation of bacteriocin production from
other cell functions. In some extensively
investigated animal-pathogen systems, this
segregation has been shown to be a natural rather
than artificially constructed separation. In some
such systems bacteriocin and/or virulence factors
have been shown to be segregated on independent
genetic elements called plasmids. In *Erwinia* the
genetic basis for bacteriocin production has not
been established; however, certain species of
Erwinia have been checked for plasmid content.
Erwinia stewartii was shown to contain between 11
to 13 plasmids ranging in size from 2.8 to 210
megadaltons (Coplin *et al.* 1980). Likewise in
Erwinia carotovora both large and small molecular
weight plasmids have been noted (Zink *et al.* 1984,
Forbes 1981). In all cases noted, the plasmids of
Erwinia have been classified as cryptic with no
attributable phenotypes. However, the maintenance
of these plasmids suggests that some yet
unidentified traits may be plasmid coded in

Erwinia.

Plasmids are defined as independently replicating extrachromosomal genetic structures (Brock 1979). The essential feature of a plasmid is that it must contain the genetic information for its own replication and maintenance, utilizing the metabolic machinery of the harbouring organism. Most plasmids can be diluted to the point of elimination using various so-called curing treatments. These approaches rely upon the fact that the elimination of most plasmids is not lethal to the host organism. Thus, plasmids code for functions that are not essential for the survival of the host. They have been isolated from a wide variety of organisms, suggesting that although plasmid-encoded features are not essential, they are probably beneficial. In evolutionary terms, the survival of the plasmid must be due to an overall increase in the fitness of the harbouring organism.

Physical evidence for the presence of a plasmid hinges upon the fact that all plasmids are substantially smaller than the circular bacterial chromosome and exist as a covalently closed

supercoiled or twisted circle (CCC). This is the most compact form within a cell; however, DNA isolation procedures can introduce a nick or break in one or both of the DNA strands. A nick in one strand results in an open circular (OC) DNA which is not supercoiled, while a nick in both strands results in a linear DNA molecule.

DNA extraction and subsequent separation of plasmid molecules involves cell lysis followed by organic extraction of protein and carbohydrates. Even the most gentle lysis protocols result in the shearing of chromosomal DNA into linear fragments. Plasmid DNA is however, largely left intact in a supercoiled conformation. Plasmid DNA can easily be separated from linear DNA using cesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation. This technique is based upon the fact that when a homogeneous solution of cesium chloride is subjected to excessive gravitational forces such as ultracentrifugation, a gradient of the salt solution is formed. This salt gradient sets up a density gradient and DNA dissolved in the salt solution is separated upon the basis of this density gradient. The incorporation of ethidium

bromide into the salt gradient serves two purposes.
Firstly, ethidium bromide intercalates into DNA and
RNA and provides a visual, reversible tag for
localization of the nucleic acids. Secondly,
ethidium bromide intercalation differentially
lowers the density of the nucleic acids. Linear
and open circular DNA bind more ethidium than
highly compact supercoiled DNA. This results in a
banding of plasmid supercoiled DNA at a higher
density than linear or open circular DNA. All
species of plasmid DNA's which are supercoiled will
band at approximately the same density.
Similarily, nicked plasmid molecules will band in
the same region as linear chromosomal DNA. Thus,
the successful separation of plasmid from
chromosomal DNA relies upon an effective
lysis/extraction protocol which minimizes the
amount of plasmid nicking.

A plasmid molecule may be nicked by physical
shearing or enzymatic cleavage. The susceptibility
of a plasmid to nicking is due primarily to its
physical size. Larger plasmids provide more sites
for enzymatic cleavage and are more susceptible to
shearing. In addition, larger plasmids are in

general under a more stringent control by the
harbouring cell and are found at a level of one to
two copies per cell (low copy number). In
contrast, small plasmids are under a more relaxed
control and are found in much higher copy number.
Furthermore, larger plasmids require certain host
components and are thus not amplified to higher
levels by the addition of a protein synthesis
inhibitor such as chloramphenicol. In striking
contrast, smaller plasmids may be amplified to
levels of one thousand copies per cell.

Although large plasmids present practical
problems with respect to their purification and
subsequent investigation, they are usually
conjugative which facilitates their rapid spread
throughout a population. In addition, large
plasmids may spend a certain proportion of time
integrated or covalently linked to the bacterial
chromosome. Subsequent conjugation and transfer of
these integrated plasmids facilitates a transfer of
both plasmid and linked chromosomal determinants.
Thus plasmids, as mobile and variable genetic
elements, provide important tools for the
understanding of prokaryotic metabolism and

regulation.

Plasmids are also important tools for the investigation of several biological and ecological phenomena. The best studied and most widespread group of plasmids contain resistance transfer factors (R factors) which confer multiple resistance to antibiotics. These R plasmids came into prominence at the end of the Second World War when antibiotics were used widely to control dysentery. Unfortunately, this created a strong selection for bacteria harbouring plasmids coding for antibiotic resistance. These R factors were able to transfer via conjugation in a surprisingly short time to various human and animal pathogens.

Plasmids are also involved in the production of various virulence factors and toxins related to disease manifestation. In some enteric pathogens, the ability to colonize the small intestine is due to the presence of a surface protein (K antigen) which is coded for by a plasmid. Alpha-hemolysin which lyses red blood cells, and enterotoxin which causes excessive secretion of water and salts are two plasmid-coded toxins produced by enteropathogenic *Escherichia coli*.

The control of both plant and animal pathogens
has involved the use of substances which inhibit
their growth. Antibiotics were first isolated from
natural inhabitants of soil. These substances had
in general a wide spectrum of activity, inhibiting
many different organisms. The effects of
antibiotics were either bacteriostatic (stopped
growth) or bacteriocidal (killed affected
organisms). The compounds themselves were simple
organic molecules which targeted primarily on the
protein-synthesizing machinery. However as
mentioned above, the widespread use of antibiotics
to combat diseases provided an extremely high
selection pressure for the development of an
efficient resistance factor. The result was the
evolution of R plasmids which usually contained
multiple drug resistance and were self-
transmissible (Broda 1979).

The basis of R factor resistance was the
production of enzymes which modified the incoming
antibiotics and rendered them useless (Brock 1979).
Similarily the solution to R factor resistance,
from a disease control standpoint, was the organic
synthesis of altered antibiotics which were not

recognized by the antibiotic-modifying enzymes. This practice set up an ongoing positive feedback cycle between chemist and pathogen. As a result, bacteriocins were considered as a possible alternative to antibiotic therapy.

In 1925, Gratia found that a certain strain of *E. coli* produced a highly specific antibiotic which inhibited another strain (Birge 1981). Further research by many workers showed that most bacteria produced proteinaceous agents which killed or inhibited closely related species. These bacteriocins, as defined by Nomura in 1967, formed a diverse group of substances frequently of high molecular weight (with respect to antibiotics), but unlike antibiotics, showed a very narrow activity spectrum.

Bacteriocin nomenclature reflects attempts to name these ubiquitous agents systematically. Individual bacteriocins are usually named after the producing organisms. For example: influenzacins are produced by *H. influenzae*, subtilins by *B. subtilis*, pyocins by *P. aeruginosa* (originally *P. pyocyanea*), colicins by *E. coli* and carotovoricins by *Erwinia carotovora*. Subclasses of bacteriocin

are identified by letters and/or numbers after the class designation. In addition, as slightly different forms of a particular bacteriocin may be produced by different strains, a complete designation also includes the strain of producer. Thus, colicin V-K357 indicates a colicin V produced by strain K357. The nomenclature is an important factor in the description of a bacteriocin as cells from a producing strain are immune to their own bacteriocin, but may be sensitive to bacteriocin produced by another species (Birge 1981).

Most bacteriocinogenic strains produce low levels under normal cultural conditions. However, bacteriocin yields may be increased from several to a thousand-fold by the same sorts of treatments which cause induction of lambda prophage (Birge 1981). Such inductions come from ultraviolet (UV) light or treatment with DNA-damaging chemicals such as mitomycin C. In some temperature sensitive or auxotrophic mutants of *E. coli*, induction can also be accomplished by heat treatment or nutrient deprivation respectively (Mayr-Harting *et al.* 1972). Parallels to phage induction can also be drawn from the fact that most producing cells

undergo lysis or quasi-lysis in releasing

bacteriocin. This is probably due to the fact that

the large size of these proteins exceeds the

functional permeability of most membranes.

Genetic analysis of bacteriocin production has

shown that these determinants are plasmid and/or

chromosomally coded (Birge 1981). Diffusible lower

molecular weight bacteriocins such as colicins

(usually less than 100 KD) were found to be coded

for by plasmids whereas, large particulate

bacteriocins (>500 KD) were found to be primarily

coded for by the bacterial chromosome. As

expected, colicins or *E. coli* bacteriocins have

been the most extensively studied group. Genetic

analysis has shown that in all cases investigated

so far, colicins have been coded for by plasmids

(Broda 1979). The plasmids fall into two major

classes. Some of these plasmids are large (greater

than 60 megadaltons), conjugative, and present in

low copy number (one to two per cell) while others

are small (less than 6 megadaltons), non-

conjugative and present in high copy numbers (10-30

per cell). Colicin-producing plasmids have always

been denoted by Col for colicin followed by an

appropriate letter. These plasmids have been made invaluable by genetic deletion and insertion of markers forming various useful cloning vehicles with colicin plasmid replicative function (col replicons).

Colicins were classified on the basis of activity aginst several *E. coli* strains. This was however complicated by the fact that most strains produced several colicins. As an alternative, colicins have been classified by activity on originally sensitive derivatives of particular strains (cross resistance grouping) (Broda 1979). Thus, derivatives of cells which are tolerant to all colicins in the cross resistance group A are designated tol A. Cells designated phage Tl resistant (tonB) or enterochelin excretion (exbB) are resistant to all colicins in the cross resistance group B. Several colicin receptors have been localized on the outer membrane and resistance to a particular colicin can in most but not all cases be traced to an alteration or loss of this receptor (Broda 1979).

Binding and subsequent action of a colicin to a sensitive cell usually results in a killing of that

cell. There are three general ways in which
colicins are known to kill cells (Broda 1979).
Some colicins act at the cell membrane and act as
energy uncouplers (col E1 and K), while others
cause a degradation of DNA (col E2) or RNA (col E3)
(Broda 1979). As mentioned above, producing cells
are immune to the bacteriocin they produce. The
molecular basis for this immunity has been
identified for both colicin E2 and E3. Cells
producing these proteins also produce a small
molecular weight immunity protein (about 10,000 D)
which complexes with each colicin in a ratio of
1:1. Dissociation of this protein from the
appropriate colicin results in an increase in the
in vitro activity of the colicin (Birge 1981).

The marked similarities in induction protocols
and assay conditions for both bacteriocins and
bacteriophage, together with their possible co-
existence in a particular cell necessitates a
method of distinction. The oldest and possibly
simplest method takes advantage of the fact that
bacteriophage, unlike bacteriocins, multiply in
their host cells resulting in localized areas of
increased titre. Thus, a series of dilutions of

the sample under test, spotted on an indicator lawn
of bacteria produces differential results for phage
and bacteriocin. Dilution of phage results in a
decreasing number of discrete phage plaques whereas
bacteriocin dilution results in a gradual thinning
of growth, which is more marked at the highest
dilutions (Mayr-Harting *et al*. 1972).

The distinction between bacteriocins and
bacteriophage became particularly difficult with *P.
aeruginosa*. This organism produces a large
particulate pyocin whose estimated molecular weight
was several million. Electron microscopy of pyocin
revealed a phage tail-like particle complete with
base plate, fibres, core and sheath. Routine spot
assays of producing strains on lawns of indicator
showed no zones of clearing due to the poor
diffusibility of pyocin. In addition, almost all
strains of *P. aeruginosa* contained one or more
bacteriophage with various degrees of defectiveness
(Mayr-Harting *et al*. 1972). These bacteriophage
tails had bacteriocidal activity, similar to pyocin
(Shinomiya *et al*, 1979). Both types of particles
were induced by standard induction regimes using
mitomycin C or UV light.

A comparative study of the so-called R-type pyocins of *P. aeruginosa* showed that all types cross reacted immunologically and appeared almost identical under EM. In addition all pyocins were shown to have a similar mode of action which resulted in a shut-down of protein and macromolecular synthesis (Ohsumi *et al.* 1980). Protein subunit composition of various pyocins was found to be almost identical except for a protein component of the tail fiber which differed in molecular weight. It has been implied that differences in this tail fiber region are responsible for the specific binding of different pyocins to the lipopolysaccharide receptors of sensitive strains (Ohsumi *et al.* 1980).

Due to resistance problems encountered in medicine, the widespread use of antibiotics in plant pathology has not been a common practice. As an alternative, the prospect for control of phytopathogenic bacteria by bacteriophage and bacteriocins was investigated (Vidaver 1976). Bacteriocins were ideal narrow spectrum specific antibacterial compounds which were less persistent, and environmentally acceptable. Unfortunately few

bacteriocins of phytopathogenic bacteria were well
characterized (Vidaver 1976). The use of agocin-84
for the control of crown gall was the only well
characterized and field tested "bacteriocin".
However agrocin-84 by definition did not fall into
the category of a bacteriocin as it was not
proteinaceous (Vidaver 1976).

In 1961 Hamon and Peron showed that *Erwinia*
carotovora subspecies *carotovora* (van Hall) Dye
produced a bacteriocin which they called
carotovoricin. An apparently similar bacteriocin
was purified to homogeneity and characterized in
1978 (Itoh *et al*. 1978). Carotovoricin-ER was
shown to be a thermolabile, particulate protein,
sensitive to sodium dodecyl sulphate (SDS),
unstable to high or low pH, but stable to
hydrolytic digestion by various proteolytic enzymes
in native conformation (Itoh *et al*. 1978).
Synthesis of carotovoricin-ER was inducible by UV
light and was accompanied by cell lysis 3-5 hours
after induction. Carotovoricin-ER visualized by EM
showed a striking resemblance to pyocin R (Itoh *et*
al. 1978, Kamimiya *et al*. 1977)

Bacteriocin addition to sensitive cells caused

a rapid and extensive lysis. This lysis was
inhibited by the addition of magnesium (Itoh *et al.*
1980). Further studies with a phospholipase A$^-$
mutant strain of *Erwinia* showed that carotovoricin-
ER binding resulted in the activation of a membrane
bound phospholipase A leading to cell lysis. This
lysis was virtually inhibited in the phospholipase
A mutant. However the cells although intact, were
still killed by carotovoricin-ER, suggesting that
phospholipase A activation is not the primary
bactericidal action of this carotovoricin (Itoh *et
al.* 1981). Carotovoricin-ER was later shown to
cause a reduction in internal ATP level which was
not due to an activation of ATPase but to an
inactivation of the energized state of the
cytoplasmic membrane necessary for ATP synthesis
and transport of amino acids (Itoh *et al.* 1982).
Essentially carotovoricin-ER reduced the proton
motive force (combination of chemical potential and
proton gradient) which drives ATP synthesis most
probably by the introduction of non-specific
membrane channels.

In order to use a bacteriocin as a possible
control agent, a thorough understanding of the

activity spectrum, mode of action and genetic and
physical basis for production is necessary. The
activity spectrum of carotovoricin-379 produced by
Erwinia carotovora subsp. *carotovora* strain 379 has
been investigated previously (Jais 1982). In
addition, the mode of action of carotovoricin-ER
had been extensively investigated (Itoh *et al.*
1980a, 1980b, 1980c, 1981, 1982). The aim of this
thesis was to study the production of
carotovoricin-379.

Specific objectives were:

1) To investigate the structural basis of
   carotovoricin-379 production using electron
   microscopy.
2) To determine if carotovoricin-379 production is
   chromosomally and/or plasmid coded.
3) To determine the serological relationships of
   the various components of carotovoricin-379.

CHAPTER 1

ULTRASTRUCTURAL EVIDENCE FOR BACTERIOCIN SECRETION

BY *ERWINIA CAROTOVORA*

INTRODUCTION

Bacteriocins are proteinaceous antimicrobial
agents produced by certain strains of bacteria
which are directed against closely related strains
(Nomura 1967). The biological role of bacteriocins
involves conferring a selective advantage to
producing strains, by killing related strains.
Some bacteriocins, such as pyocins, have highly
complex protein structures which resemble
bacteriophage tails. In fact, most bacteriocins
are induced by prophage-inducing agents such as
mitomycin C or ultraviolet irradiation. Like
lambda prophage, bacteriocins may be released
either by a lysis, or quasi-lysis mechanism
depending on the molecular weight of the protein
and inherent permeability of the producing strain
(Birge 1981). These observations imply that
bacteriocin production may have evolutionary links

to defective phage components.

Bacteriocin production by *Erwinia carotovora*, which causes potato blackleg and soft rot of a variety of economically important agricultural crops, was first shown by Hamon and Peron in 1961. Subsequently they coined the term carotovoricin for bacteriocins produced by *Erwinia carotovora* (*Ecc*). Further research has demonstrated the existence of two types of bacteriocin activity in some strains. One is characterized by large diffuse zones of inhibition and the other by small clear zones of inhibition (Crowley and DeBoer 1980, Jais 1982). Physical characterization of purified carotovoricin preparations having the latter activity established that thermolabile, particulate, trypsin-resistant proteins were involved (Itoh *et al.* 1978). Phage tail-like particles with a contractile sheath, core, base-plate and fibres were observed by electron microscopy (EM) (Itoh *et al.* 1978).

Initial work with carotovoricins implied that release of these bacteriocins is due to cell lysis following induction. However, some strains of *Erwinia* produce substantial amounts of carotovoricin constitutively with no significant

reduction in turbidity or cell viability.
Moreover, lysis was detected only after induction
by mitomycin C or ultraviolet irradiation and
following an increase in carotovoricin titers (Itoh
et al. 1978). These observations suggested that
possibly some mechanism other than cell lysis was
also involved in carotovoricin liberation.

In this study, carotovoricin-379 production in
Erwinia carotovora subspecies *carotovora* strain 379
(*Ecc* 379) was investigated by ultrastructural
examination of bacteriocin-producing cells at
various times after induction. The observations
made support the hypothesis that the particulate
component of carotovoricin-379 is secreted on
membrane enclosed, fimbrae-like projections, the
tips of which swell to form detachable vesicular
heads.

MATERIALS AND METHODS

Culture of *Erwinia carotovora* Strains and Induction of Bacteriocin Production:

Erwinia carotovora subsp. *carotovora* strain 379 was chosen as a bacteriocin producer. This strain constitutively produces particulate bacteriocin but can be induced to produce it at much higher titres. Three strains of *E. carotovora* subspecies *atroseptica* and one strain of *E. carotovora* subsp. *carotovora* were used as indicators (Table 1). Bacteria were grown in Luria Broth (Maniatis *et al.* 1984) pH 7.4 or minimal M9 medium pH 7.4 (Maniatis *et al.* 1984) at 20 C on a rotary shaker (100 rpm). Enhanced bacteriocin production was induced by the addition of mitomycin C at 0.2 ug/ml 2 hours (h) after inoculation.

Bacteriocin Plate Assays:

Cultures were vortexed at high speed for 30 seconds (s) at 8, 12 and 24 h after induction, and supernatants were viewed by electron microscopy (EM) as described below. Bacteriocin activity was

Table 1: Carotovoricin producing and sensitive strains of *Erwinia carotovora*

Producer:

Erwinia carotovora subsp. *carotovora* strain 379
    serogroup XI

Sensitive strains:

Erwinia carotovora subsp. *atroseptica* strain SR8
    serogroup I
Erwinia carotovora subsp. *atroseptica* strain 496
    serogroup XXII
Erwinia carotovora subsp. *atroseptica* strain 530
    serogroup XX
Erwinia carotovora subsp. *carotovora* strain 504
    serogroup XVIII

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assayed by placing 5 ul of test samples on a lawn of indicator strains seeded in peptone soft agar (0.85% NaCl, 1% Bacto-Peptone, 0.45% Bacto-Agar pH 7.4).

Concentration of Carotovoricin-379:

Aliquots of induced cultures were pelleted at 10,500 x g for 20 min. The supernatant fractions were briefly vortexed (15 s at high speed) and filter-sterilized using a 0.22 um filter. Sterilized 18% (w/v) polyethylene glycol (PEG) M.W. 8000 was dissolved in the sterile supernatants at 22 C and the mixtures were allowed to stand on ice overnight to facilitate precipitation. The precipitates were collected by centrifugation at 13,500 x g for 25 min and the pellets were dissolved in 1/100 the original volume of 50mM sodium phosphate buffer pH 7.4. Insoluble material was removed by low speed centrifugation at 8000 x g for 10 min at 4 C. The resulting carotovoricin-379 preparations were analysed by column chromatography or electron microscopy.

Column Chromatography:

Concentrated carotovoricin-379 (0.5 ml) was applied to the top of a 35 cm x 1.8 cm column packed with Sepacryl S-300. The running buffer was 50mM sodium phosphate pH 7.4 and the flow rate was maintained at about 0.5 ml/min. Eluate was monitored at 280 nm, and peaks were collected and bioassayed as described above.

Electron Microscopy:

Concentrated carotovoricin-379 (10 ul) was loaded onto copper grids coated with collodion-carbon and incubated at room temperature for 3 to 5 min. Grids were then stained with 10 to 12 drops of 2% phosphotungstic acid (PTA) pH 7.0, allowed to dry, and viewed in a Phillips EM-300 electron microscope (Hill 1984).

Modified Negative Staining of Whole Cells:

In order to increase the resolution of negatively stained whole cell preparations, two protocols for cell pretreatment were used. In the first procedure, cultures were prefixed in 0.2% osmium tetraoxide (final concentration added

directly to growth media) for 1 h at 20 C, washed thrice with sterile broth and stained with 2% PTA pH 7.0.

In the second pretreatment procedure, reagent grade toluene was added at 1:2 (v/v) to an aliquot of bacterial cells. The tubes were capped and gently inverted 10 times allowing complete separation of the two phases after each inversion. The cell suspension was then removed and stained as before with PTA.

Fixation and Embedding:

Cells were fixed in agar according to the Ryter-Kellenberger procedure (1958) using 1% osmium tetroxide in veronal acetate buffer supplemented with calcium chloride (Kellenberger buffer), for 14 to 16 h at room temperature. Blocks of cells were subjected to a serial alcohol dehydration and embedded in EPON. Thin sections were stained for 15 to 20 min in 5% uranyl acetate.

RESULTS

Mitomycin C Induced Cultures:

Ultrastructural examination of induced cultures of *E. carotovora* subsp. *carotovora* after 48 h at 20 C showed bacterial cells with a proliferation of fimbrae-like projections of various lengths (Fig. 1). Low levels of particulate carotovoricin-379 were seen in close association with intact bacterial cells (Fig. 1d). Thin sections of induced cells revealed projections with a subunit structure at various stages of elongation (Fig. 1c). In some preparations, vesicular projections were also present in addition to fimbral projections (Fig. 1a-b).

Examination of the Supernatant Fractions:

Filter sterilization (0.22 um filter) of supernatants from induced cultures markedly reduced bacteriocin activity. However, a brief (15 s) shearing by vortex before filtration resulted in a retention of activity. Microscopic examination of non-filter-sterilized preparations showed that

Figure 1. Electron micrographs of mitomycin C
induced cultures of *Erwinia carotovora* subsp.
carotovora strain 379 48 h after induction at
20 C (Bars=100nm).
   a-b.   Negative stained cells showing both
          vesicular and fimbrae-like projections;
      c. Thin section of a producing cell showing
          subunit structure of fimbrae-like project-
          ions at various stages of elongation.
          Insert: area containing early stages of
          projection;
      d. Negative stained cell showing fimbrae-like
          projections and close association of
          particulate carotovoricin with intact
          cell.
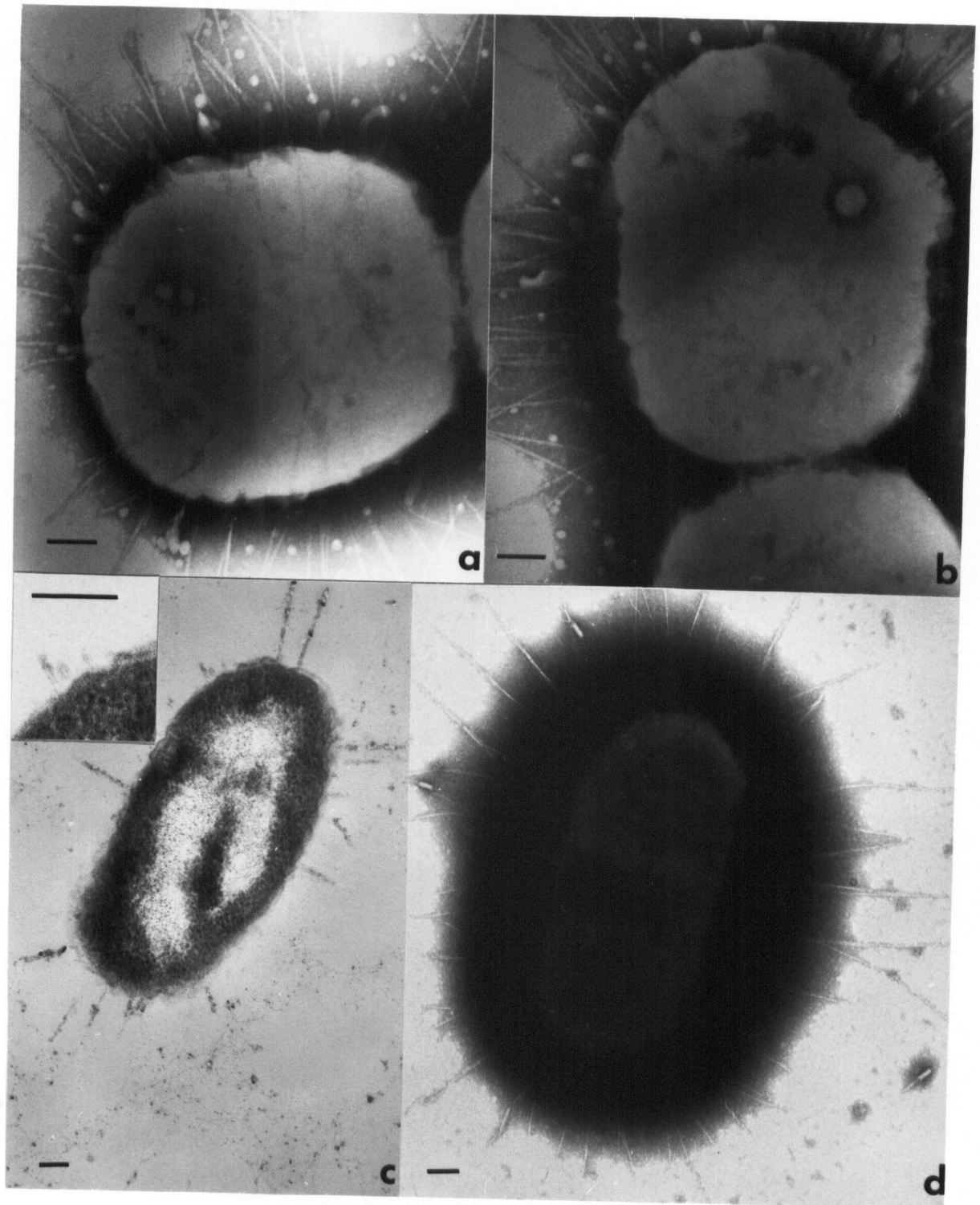
carotovoricin particles were aggregated and
physically attached by a central core to vesicles
(Fig. 2a) This observation explained the loss of
activity upon sterilization as vesicles and
attached carotovoricin-379 would be retained on the
filter. The brief shearing presumably detached
these particles. Vesicles often had long thread-
like projections attached to them which were of
exactly the same diameter as carotovoricin cores
(Fig. 2b).

**Serial Subunit Structure of Particulate
Carotovoricin-379:**

Carotovoricin particles consisted of subunits
arranged around a central core which extended
through the sheath (Fig. 2c-g). In rare instances
the core was attached terminally to a vesicular
head (Fig. 2g). Although attached heads were
uncommon, numerous detached heads were found in
concentrated preparations suggesting a rather weak
association with the core (Fig. 2g).

Figure 2. Negative stained preparations of particulate carotovoricin-379 concentrated from the supernatant fraction of mitomycin C induced *Erwinia carotovora* subsp. *carotovora* strain 379 (Bars=100nm).

a. Carotovoricin-379 attachment to vesicles by central core;
b. Vesicle with long core-like projections attached;
c. Contracted form of carotovoricin-379 showing core surrounded by sheath. Note diagonally displaced, disrupted particle illustrating a central core with a modular arrangement of surrounding sheath;
d. Partially disrupted carotovoricin-379 sheath exposing a connecting core. Note the oval upper terminus of the core;
e. Carotovoricin-379 particle with an intact sheath surrounding the lower half and a partially disrupted sheath surrounding the upper half. This disrupted sheath partially obscures a central core which terminates in a block-like structure;
f. Two separated sheath components each containing a protruding core;
g. Contracted form of carotovoricin-379 showing attached head. Attachment is facilitated by stem-like projections. Note numerous free floating vesicular heads.
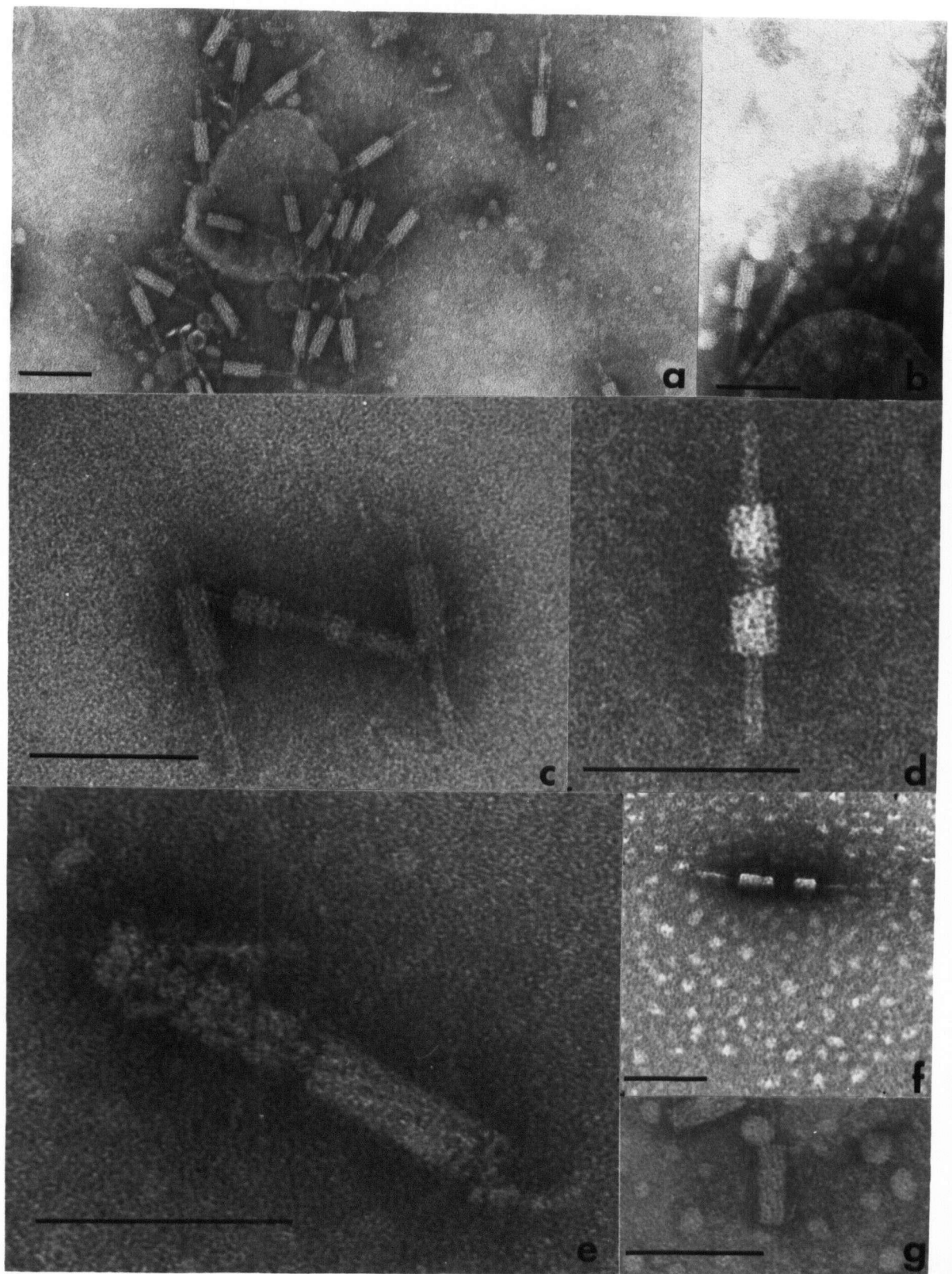
Developmental Stages of Carotovoricin-379

Production by Mitomycin C Induced Cells:

Particles found in the concentrated

supernatants 8 h after induction appeared to be

immature carotovoricin on the basis of appearance

and activity on known indicators strains (Fig. 3a-

f). In some cases these immature particles were

fully encapsidated while others had a definite

swelling (Fig. 3b). Other particles were open at

both ends suggesting intermediate positions on a

bacteriocin particle (Fig. 3c and d). These

particles were observed to aggregate end to end at

a low frequency, suggesting a serial association

(Fig. 3d).

Particles found in 12 h preparations resembled

mature carotovoricin-379. They were larger in

diameter and more elongate than 8 h particles with

a definite beaded structure (Fig. 4a). At this

stage, cores in the process of thickening were

observed (Fig. 4b). Whole cell preparations were

also observed with released particles, or partially

formed particles with subunit addition to surround

and enclose the numerous projecting core filaments

(Fig. 4c). Fully mature particles were released

Figure 3. Negative stained immature
carotovoricin-379 sheared from producing cells of
Erwinia carotovora subsp. *carotovora* strain 379 8 h
after mitomycin C induction and the plaque types
they induce in sensitive indicator strains.
  a. Negative stained particles found in
      supernatant (Bar=100nm);
  b. Immature particles showing terminal
      vesicular swelling and partial encapsidation
      (Bar=100nm);
  c. Sheath-like particle without projecting core
      (Bar=10nm);
  d. Two sheath-like particles as seen in 3c
      linked end-on (Bar=10nm);
  e. Typical clear plaques produced by large
      molecular weight carotovoricin-379 (Seph-
      acryl S-300 peak 1);
  f. Typical diffuse plaques produced by low
      molecular weight immature carotovoricin-379
      (Sephacryl S-300 peaks 2 to 4).
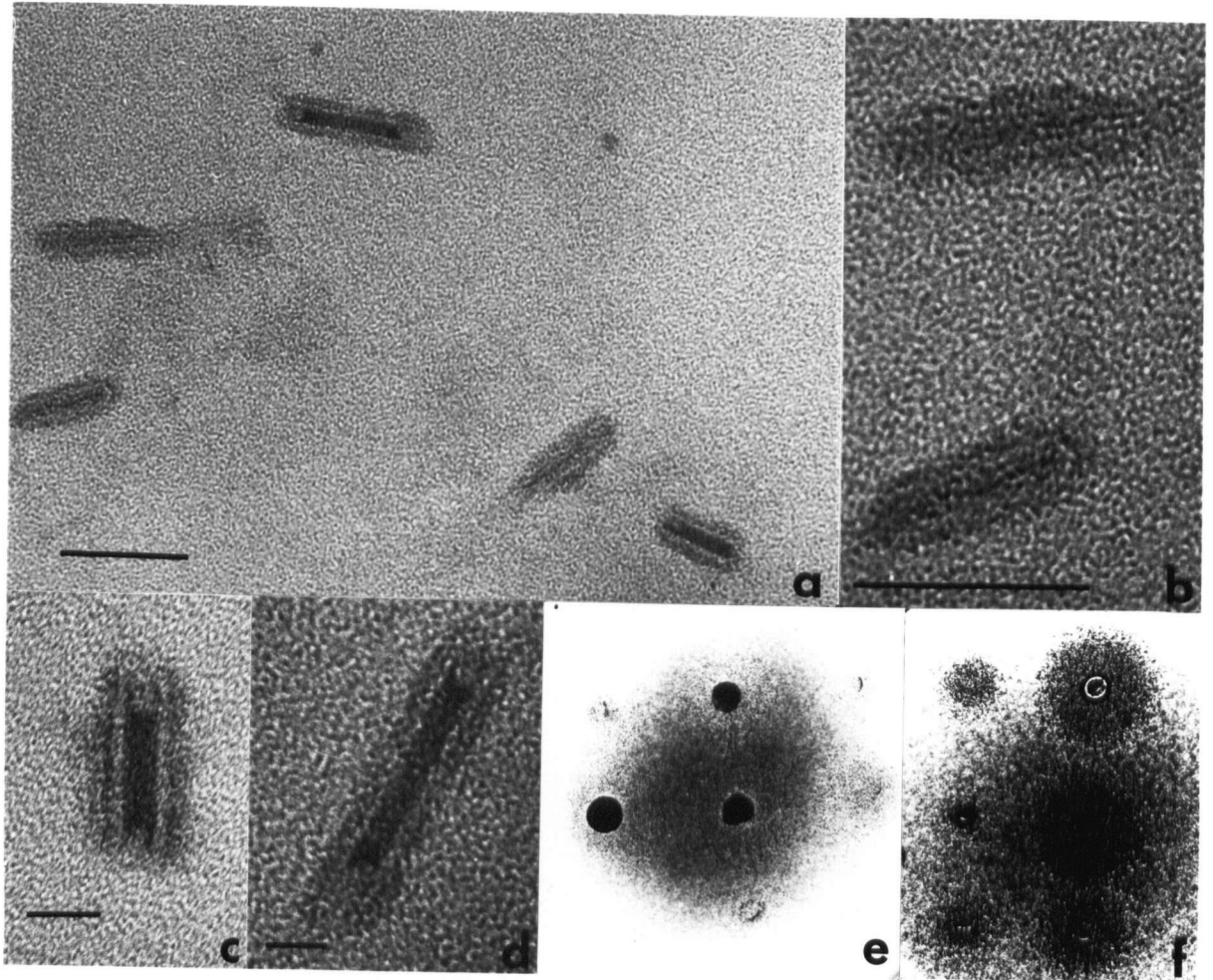
Figure 4. Negative stained carotovoricin-379 particles and cellular projections produced by mitomycin C induced cultures of *Erwinia carotovora* subsp. *carotovora* strain 379. Culture fluids were either negatively stained with 2% phosphotungstic acid pH 7.0 (a to d), or pretreated with 0.2% osmium tetroxide (e) or an equal volume of toluene (f to i) prior to negative staining. All bars = 100 nm.

  a. Carotovoricin-379 particles 12 h after induction which appear elongated and thickened due to the addition of material to the developing central core;
  b. Isolated central core in the process of thickening;
  c. Carotovoricin-379 particles either released (left) or in the process of thickening (right) in association with intact cells 12 h after induction;
  d. Fully mature carotovoricin-379 particles released 24 h after induction with contracted (left) or extended (right) sheaths;
  e. Intact mature carotovoricin-379 particle with attached head protruding from a producing cell;
  f. Core-like projection with terminal vesicle attached to bacterial cell 8 h after induction;
  g. Bacteriocin-like projection attached to a producing cell 12 h after induction;
  h. Particulate carotovoricin-379 attached to and released from an intact producing cell 16 h after induction;
  i. Protruding particulate carotovoricin-379 with attached head surrounded by intact outer membrane 24 h after induction.
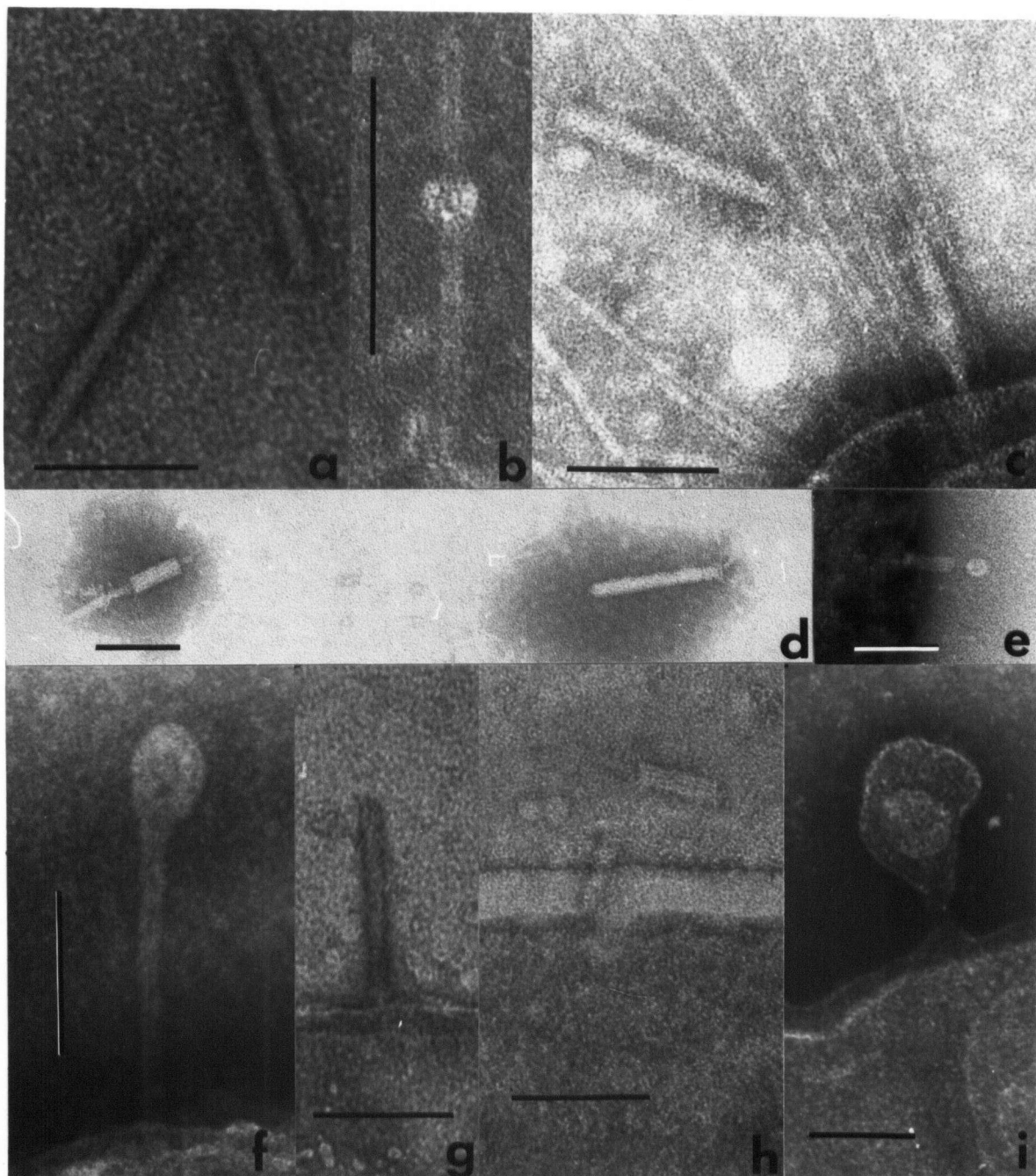
into the supernatant and were readily detected in contracted or extended forms 24 h after induction (Fig. 4d).

Extrusion of Carotovoricin through the Membrane:

The modified staining procedure using osmium tetroxide provided more detail and clearly showed carotovoricin-379 particles with attached heads extruded through the membrane (Fig. 4e). The disadvantage of the procedure was that the washings resulted in a loss of bacterial surface appendages (Fig. 4e) and partial disruption of bacteriocin particles.

Bacteriocin-like projections at 8, 12, 16 and 24 h respectively, after induction (Fig. 4f-i) were visualized with the modified staining procedure using a toluene pretreatment. These particles corresponded well in appearance and dimensions with the immature particles isolated in the sheared supernatant preparations described previously. However, these preparations showed physical association of bacteriocin intermediates with intact cells. Pretreatment with toluene presumably partially disrupted the outer membrane allowing

penetration of PTA. As no washing steps were

necessary, bacterial surface ultrastructure

remained intact.

Sephacryl S-300 Column Chromatography:

Gel filtration of concentrated carotovoricin-

379 harvested at 44, 52, 58 and 62 h after

induction showed a definite shift to higher

molecular weight bacteriocin (Fig. 5). At 44 h,

two major peaks corresponding to globular protein

molecular weights of 300,000 daltons (peak 1) and

20,000 daltons (peak 2) respectively had

bacteriocin activity. In addition a minor peak

equivalent to 15,000 daltons (peak 3) was present

and had bacteriocin activity. This minor peak

appeared with an even smaller molecular weight peak

(about 10,000 daltons) (peak 4) at 52 h after

induction. At this time peak 2 was almost at the

level of peak 3 and peak 1 had doubled in

concentration. This general trend was seen in the

shift from 58 to 62 h after induction with the

appearance of still another bacteriocin peak (1b)

corresponding to 180,000 daltons at 62 h.

Bioassays of these fractions detected bacteriocin
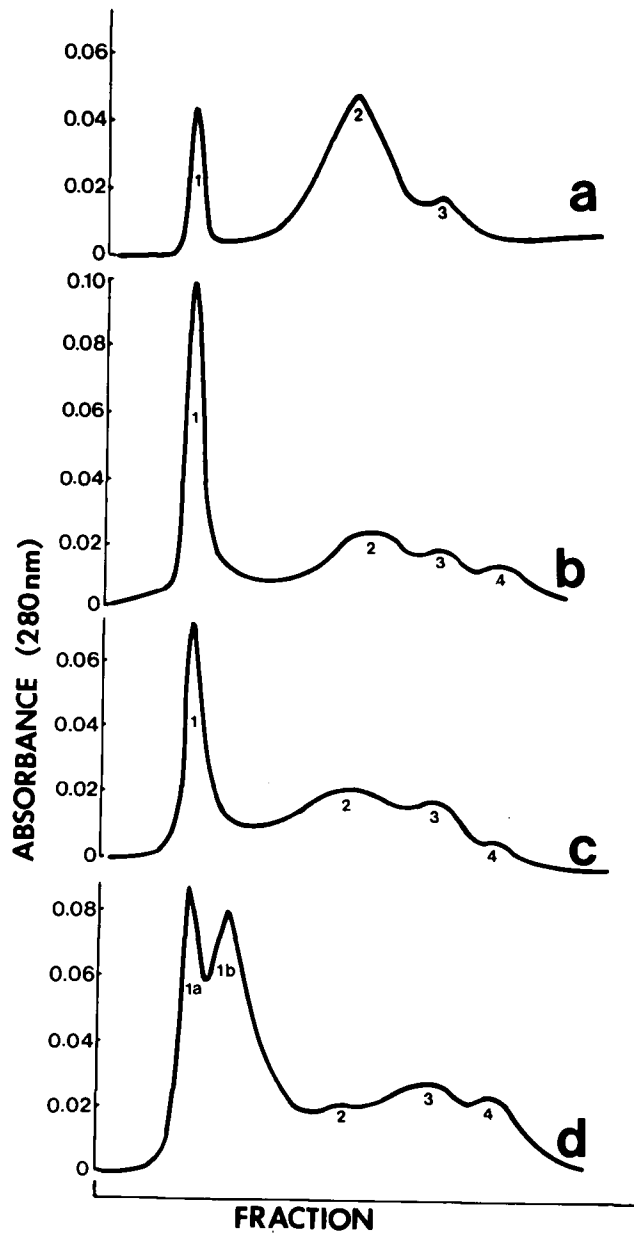
Figure 5. Sephacryl S-300 column chromatograms of carotovoricin-379 at (a) 44, (b) 52, (c) 58 and (d) 62 h after mitomycin C induction in concentrated supernatants of *Erwinia carotovora* subsp. *carotovora* strain 379.

activity with different degrees of clearing
depending on the indicator strain used. Strain SR8
consistently showed the greatest sensitivity to all
peaks. All strains of indicator were most
sensitive to peaks 1a and 1b and produced clear
zones of inhibition (Fig. 3e). The low molecular
weight peaks gave varying degrees of diffuse
inhibitory zones (Fig. 3f) on all indicators except
strain 530 which was resistant to low molecular
weight carotovoricin-379.

Electron micrographs of the separated
bacteriocin components indicated that as subunits
were added to bacteriocin particles over time,
there was an increase in molecular weight (Fig. 6).
However, the existence of a soluble low molecular
weight component was still possible as peak 4
showed diffuse-type activity on strain SR8, but no
particulate structure could be resolved by electron
microscopy.

Induced cultures which were re-induced and
diluted with an equal volume of medium (to maintain
a mitomycin C concentraion of 0.2 ug/ml) showed
that all peaks in Figures 5a-d were present in a
single scan at 8 h after the second induction (Fig.

42

Figure 6.   Sephacryl S-300 column chromatograms of
carotovoricin-379 produced by *Erwinia carotovora*
subsp. *carotovora* strain 379 48 h after mitomycin C
induction and electron micrographs of the
corresponding negative stained peak contents.
Bar=100nm.

43
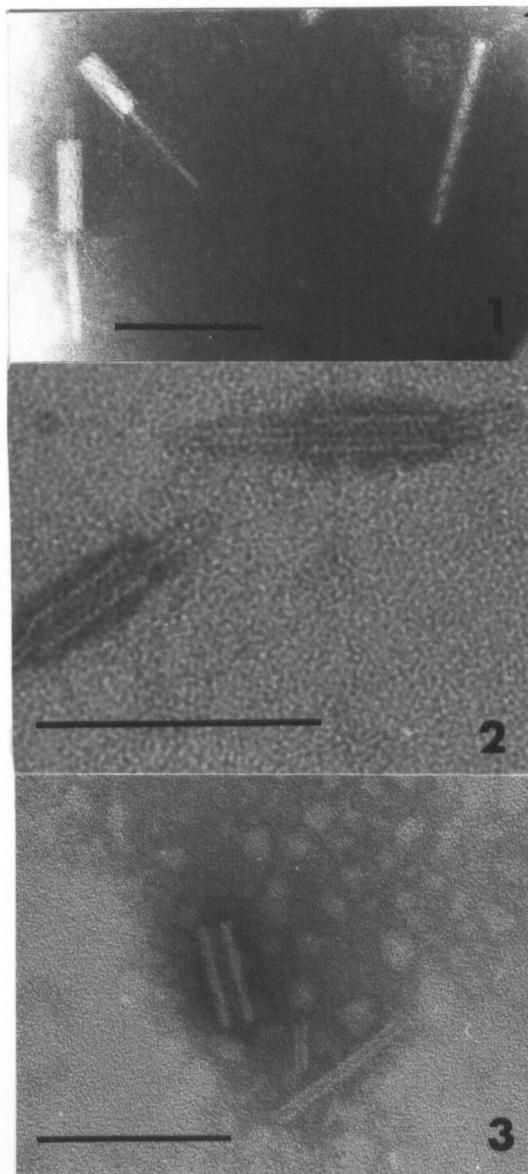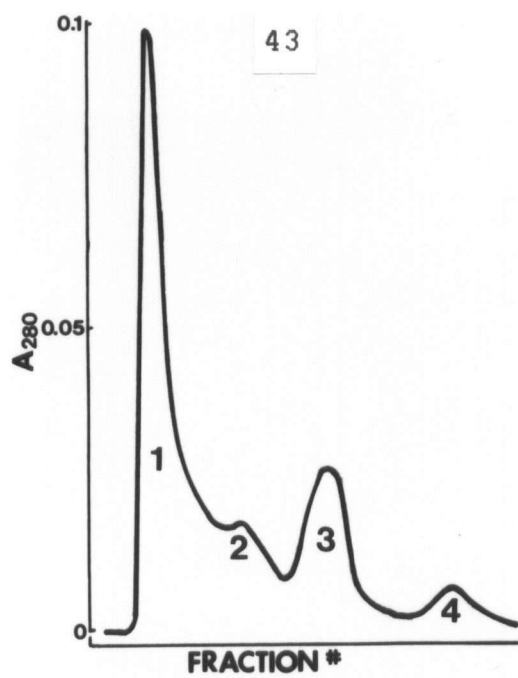
7a). This suggested that the second induction
initiated another round of carotovoricin-379
production which appeared as satellite peaks on the
S-300 chromatogram (Fig. 7a).

All supernatant bacteriocin activity was
detected in one major peak when 20mM magnesium
sulfate was added to the medium followed by
induction (Fig. 7b). This peak profile was
identical to the profile of carotovoricin-379
concentrated from induced cells grown in minimal M9
media which also contained magnesium (Fig. 7c).

DISCUSSION

Cell lysis, as seen by a reduction in
turbidity, occurred in induced cultures of *Erwinia
carotovora* subsp. *carotovora* strain 379 (*Ecc* 379)
36 h after induction. However carotovoricin
particles were seen in culture supernatants and
bioassayed at 8 h after induction. Although no
cell lysis or reduction in cell viability was
detected in non-induced cultures, bacteriocin
production was easily detected by bioassays and
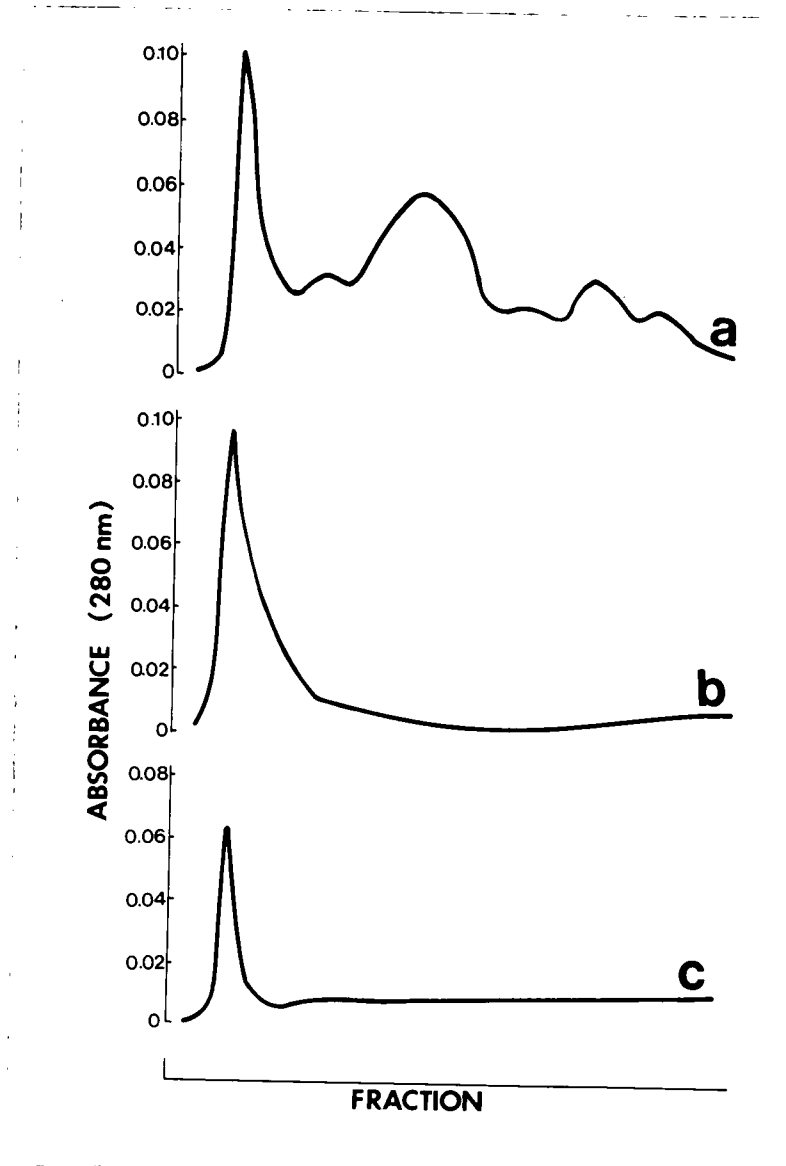
Figure 7. Sephacryl S-300 chromatograms of carotovoricin-379 produced by *Erwinia carotovora* subsp. *carotovora* strain 379 in mitomycin C induced cultures grown under different conditions.

    a.   Effect of a second induction after 8 h with mitomycin C in Luria broth (no added magnesium);

    b.   Effect of the addition of 20mM magnesium sulphate to Luria broth prior to induction;

    c.   Effect of magnesium present as a component of minimal M9 media.

electron microscopy.

Cell lysis following induction is a common feature in the production of large and small molecular weight bacteriocins and is similar to temperate phage induction. Recent work with colicins has shown that lysis is dependent on expression of a lysis gene which activates a phospholipase altering the membrane permeability. This gene product may be directly involved in membrane transport of colicin. The effect on cell integrity by the lysis gene can be inactivated by incorporation of 20mM magnesium in the medium (Pugsley and Schwartz 1984).

In our experiments, addition of 20mM magnesium to the culture media followed by induction delayed lysis by 6-8 h. Under these conditions, gel filtration of carotovoricin-379 containing supernatants showed a loss of smaller molecular weight, less active peaks. All carotovoricin activity was detected in the large molecular weight fraction. The magnesium ions probably stabilized the outer membrane sufficiently to prevent release of partially active bacteriocin intermediates. These observations suggest that in addition to cell

lysis another mechanism may be involved in bacteriocin release.

Erwinia carotovora subsp. *carotovora* produces a wide variety of pectolytic and cellulolytic enzymes. There have been several reports of a simultaneous induction of pectin lyase and particulate carotovoricin or temperate bacteriophage in *Erwinia carotovora* (Chatterjee 1984, Itoh *et al.* 1980 and Kamimiya *et al.* 1977). These enzymes and carotovoricins appear externally in the supernatant fraction of cultured cells and co-induction could be explained by an inactivation of a common repressor of an existing secretory system.

The outer membrane of the Enterobacteriaceae provides an effective barrier to the release of substances > 700-1000 daltons (Nikaido and Vaara 1985). Thus, there is a need for a secretory system for exo-enzymes. The secretion of enzymes from *Pseudomonas* and endotoxin production by *Neisseria* is thought to occur via the formation and subsequent release of cell wall blebs or outer membrane vesicles (Devoe *et al.* 1973). These blebs are similar to those observed in this study. In

conjunction with the numerous fimbrae-like
projections, these blebs may form a secretory
mechanism for bacteriocin and possibly macerating
enzymes.

On the basis of ultrastructural observations
and experimental data we propose the following
hypothetical model for carotovoricin-379 assembly
and secretion (Fig. 8). The construction of
bacteriocin occurs within an outer membrane vesicle
or "bleb". This vesicle provides a general
mechanism for secretion of all *Erwinia* exoproteins.
Macerating enzymes and carotovoricin components are
produced in the cytoplasm and are transported
across the plasmalemma probably by the model of
Randall and Hardy (1984). The secretion of these
proteins is facilitated by their exocytosis within
an outer membrane vesicle. Carotovoricin-379
construction occurs within a vesicle which also
contains a fimbrial projection. This fimbrial
projection ultimately becomes the core of
carotovoricin-379. Some bacteriocin proteins are
added externally to the fimbrial projection
resulting in the formation of a contractile sheath.
Other bacteriocin proteins are transported into the

Figure 8. Hypothetical model for the production of carotovoricin by *Erwinia carotovora* subsp. *caroto-vora* strain 379.

   a. *Erwinia* exoproteins (macerating enzymes and carotovoricin) are produced in the cytoplasm and are transported across the cytoplasmic membrane (cm) into the periplasmic space (ps). These proteins are subsequently secreted within an outer membrane vesicle (omv).

   b. Carotovoricin construction occurs inducibly within an outer membrane vesicle which also contains a fimbrae-like projection (a). Under non-induced conditions these projections extend outwards and form the numerous fimbrae present on carotovoricin producing strains.

   c. Under induction, carotovoricin components translocated into the periplasmic space are added to the fimbrae-like projection. These added components eventually form the sheath(s) of the particulate carotovoricin. During the final stages of construction, carotovoricin components responsible for the formation of a baseplate (bp) are added at the base of the carotovoricin particle.

   d. Some carotovoricin components may be translocated through the central core to an expanding terminal vesicle or head (h).

   e. Release of the carotovoricin is facilitated by the activation of a phospholipase which breaks open the enclosing outer membrane releasing intact carotovoricin with tail fibres (tf) and base plate (bp).

   f,g. The head (h) of the carotovoricin may dissociate from the rest of the particle in the supernatant of a producing strain. The rest of the carotovoricin may exist in two forms either extended (F) or contracted (G).
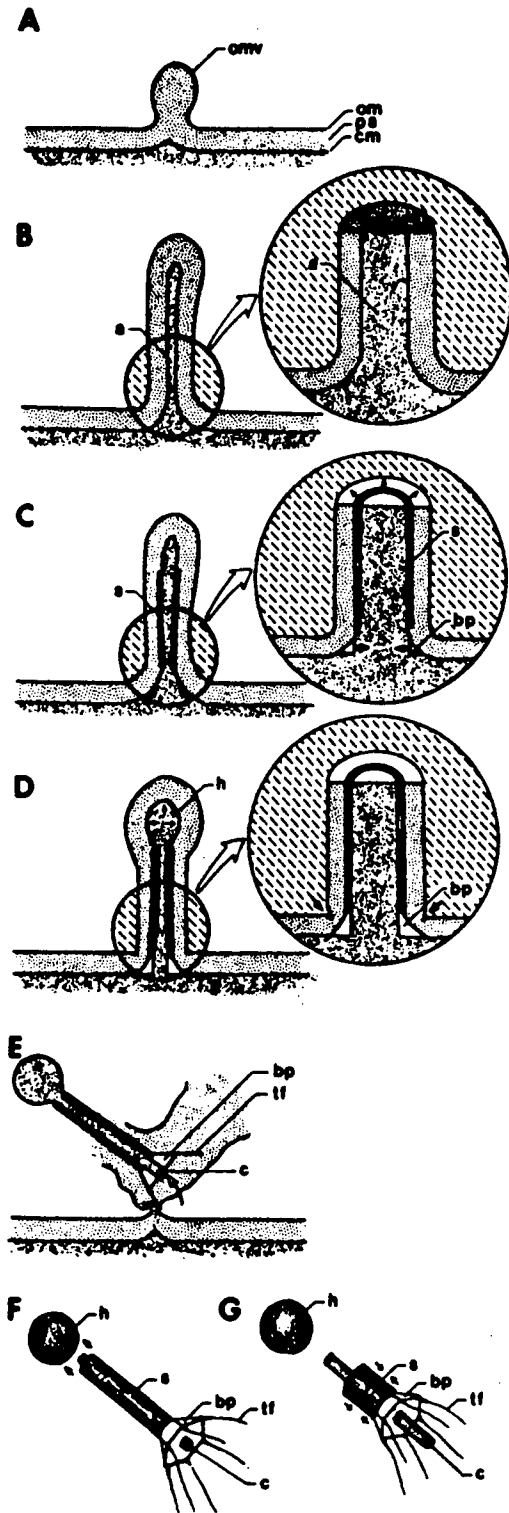
hollow core and subsequently translocated to a terminal vesicle. Upon completion of an intact carotovoricin particle, it is released within a vesicle. The release of carotovoricin followed by disruption of the vesicle may be associated with the activation of a phospholipase. This phospholipase may be found associated with the bacteriocin or may be activated within the periplasm by some induced bacteriocin component. DNA damage by chemical or physical agent activates the production of recA protein which deactivates a repressor (Glass 1985) of both bacteriocin and lytic components. This results in an increase in bacteriocin construction and secretion which, along with the activation or production of a phospholipase, results in the disruption of the producing cell.

In the non-induced state the construction and secretion of the bacteriocin and some of its components are repressed; however, the exocytosis of periplasmic proteins is still active resulting in a secretion of partially active bacteriocin components as well as the secretion of macerating enzymes normally found in the periplasm. The

repression however is not complete resulting in a
differential low level of non-induced particulate
bacteriocin production.

CHAPTER 2

GENETIC DETERMINANTS OF CAROTOVORICIN PRODUCTION

IN *ERWINIA CAROTOVORA*

INTRODUCTION

Bacteriocins are proteinaceous antimicrobial
agents produced by certain strains of bacteria
which are active only against closely related
strains (Nomura 1967). Bacteriocin production is
common in a number of genera and is generally
considered to confer a selective advantage to
producing strains (Birge 1981). Bacteriocin
production in *Erwinia carotovora*, which causes soft
rot of vegetables and blackleg of potato was first
described by Hamon and Peron (1961). Subsequent
research resolved two types of bacteriocin. The
first is characterized by a small clear zone of
inhibition and the second by a large and diffuse
zone. A thermolabile, trypsin-resistant, phage
tail-like particulate bacteriocin, called
carotovoricin-ER, was identified as causing the

small clear zones (Itoh *et al.* 1978). This
bacteriocin resembled the pyocins produced by
certain *Pseudomonas* strains and like the pyocins
was inducible by ultraviolet irradiation, mitomycin
C or other DNA damaging agents (Birge 1981).
Recent investigations have shown that both pectin
lyase activity and bacteriocin activity are co-
inducible in *E. carotovora* (Kamimiya *et al.* 1977)
and in *E. chrysanthemi* (Chatterjee *et al.* 1984).

Genetic analysis of bacteriocin production in
other genera has shown that the determinants can be
plasmid or chromosomally encoded (Birge 1981). Low
molecular weight bacteriocins causing large diffuse
zones of inhibition, such as the colicins were
found to be plasmid encoded. Particulate
bacteriocins causing small clear zones of
inhibition were primarily coded for by the
bacterial chromosome. Although both small and
large plasmids have been isolated from several
different strains of *Erwinia carotovora* (Coplin *et
al.* 1980, Forbes 1981, Zink *et al.* 1984), no
phenotypes could be assigned and they were
concluded to be cryptic.

Previous work in this laboratory has shown that

both types of bacteriocin activity are associated

with *Ecc* strain 379 (Jais 1982) and that the

bacteriocin is produced in intimate association

with intact cells possibly via extrusion (Chapter

1). The purpose of this study was to determine

whether the determinants for bacteriocin production

in *Ecc* strain 379 were chromosomally and/or plasmid

encoded.

MATERIALS AND METHODS

Media and Growth Conditions:

All strains (Table 1) were grown in Luria Broth

(LB) pH 7.4. *Erwinia carotovora* was incubated at

20 C while *Escherichia coli* was grown at 37 C.

Antibiotics obtained from the Sigma Chemical

Company were dissolved in water or alcohol and used

in plates or liquid culture at the recommended

concentration (Maniatis *et al.* 1982). Erythromycin

was dissolved in alcohol and added to LB pH 8.0 at

a final concentration of 30 ug/ml.

Crystal violet pectate (CVP) media was made

Table 1. Bacterial strains used
_____

Carotovoricin Producer:

Erwinia carotovora subsp. *carotovora* strain 379
    serogroup XI

Carotovoricin Indicators:

Erwinia carotovora subsp. *atroseptica* strain SR8
    serogroup I
Erwinia carotovora subsp. *atroseptica* strain 530
    serogroup XX
Erwinia carotovora subsp. *atroseptica* strain 496
    serogroup XXII
Erwinia carotovora subsp. *carotovora* strain 504
    serogroup XVIII

Other Strains:

Escherichia coli strain HB101 (virgin)
Escherichia coli strain HB101 + plasmid pBR322
Escherichia coli strain HB101 + plasmid R68.45

_____

according to Cuppels and Kelman (1974). CVP (20 ml) plates were spread with appropriate volumes of antibiotic stock solution to give the recommended final concentration. Passive diffusion for 24 hours (h) at room temperature was allowed before use. Using this same technique, CVP + 1% LB was prepared to accelerate growth of *E. coli*.

Mating Protocols:

All matings were performed at room temperature. R68.45 was transferred from an *E. coli* harbouring strain to *Erwinia carotovora* subsp. *carotovora* strain 379 using a standard plate mating technique (Puhler and Riess 1984) and transconjugants were selected on CVP + kanamycin (50 ug/ml). These transconjugants were mated back to virgin *E. coli* strain HB101 and transconjugants were selected on LB + kanamycin (50 ug/ml) + streptomycin (25 ug/ml).

For wild-type matings, (without R68.45), 1 ml of a mid-log phase culture of *E. carotovora* subsp. *carotovora* strain 379 and *E. coli* strain HB101 (with and without PBR322) were added to 20 ml of sterile LB. These mixtures were allowed to stand

at room temperature for 4 h. Aliquots of cells

were selected on LB + erythromycin (30 ug/ml) +

streptomycin (25 ug/ml). All transconjugants were

tested for: ability to produce carotovoricin-379,

ability to grow on CVP and resistance to

erythromycin (30 ug/ml) and chloramphenicol (15

ug/ml).

Bacteriocin Plate Assays:

Assays were performed by placing 5 ul filter-

sterilized culture supernatant, or contents of a

peak from a column, on a lawn of indicator seeded

in peptone soft agar (PSA) containing 0.85% NaCl,

1% Bacto Peptone, 0.45% Bacto Agar pH 7.4.

Carotovoricin concentration, column

chromatography and electron microscopy were

executed according to procedures outlined in

Chapter 1.

Total DNA Extraction and Electrophoresis:

Late log phase cells (10 ml) were harvested by

centrifugation and resuspended in 600 ul of 0.05M

Tris, 0.02M EDTA; pH 8.0 (TE) buffer + 20% sucrose.

One hundred microliters of a 5 mg/ml stock solution

of lysozyme (Sigma) in TE was added and incubated
for 10 minutes (min) at room temperature. One
hundred microliters of a 0.5M EDTA stock solution
pH 8.0 were then added and incubated for 20 min at
room temperature. Two hundred microliters of a 10%
stock solution of SDS were then added. The tubes
were gently inverted 10 times and incubated at 50 C
for 20 min. This lysate was extracted twice with
phenol:chloroform (50:50 v/v) and twice
subsequently with chloroform, until a clear
interface was seen (Maniatis *et al*. 1982). The
aqueous phase was adjusted to 0.1M NaCl from a 5M
NaCl stock solution, and DNA and RNA were
precipitated with 2 volumes of 99% ethanol at -50 C
overnight.

Precipitated nucleic acids were pelleted at
13,000 x g at -20 C for 25 min. Residual ethanol
was removed in a vacuum dessicator and the pellet
was dissolved in 50 ul of TE. Ten microliter
aliquots of these samples were removed, treated
with 2 ul of DNase-free RNase (10 mg/ml stock), for
30 min at 37 C and analysed by agarose gel
electrophoresis (AGE) (Perball 1984; Hames *et al*.
1984; Glover 1985).

Electrophoresis was performed on a homemade 20 x 20 cm submerged, horizontal apparatus. Electrophoresis was carried out in 90mM Tris, 90mM Borate, 2.5mM EDTA (TBE) pH 8.2 at 1.5 V/cm for 18-24 h. Gels used were 0.5% agarose (w/v) (Sigma), poured to a depth of 0.3 mm. DNA was stained in 1.0 ug/ml of ethidium bromide (Sigma) for 1 h and viewed with a mid-wave ultraviolet transilluminator (U.V. Products Inc. San Gabriel, California).

Alkaline phosphatase was assayed by combining 100 ul of filter-sterilized supernatant with 100 ul of substrate buffer (0.1M Tris-HCl pH 9.0) containing 1 mg/ml p-nitrophenyl phosphate (Sigma) in 1.5 ml polyallomer Eppendorf tubes. Tubes were incubated at 4 C overnight. Positive results were identified by a visual detection of a yellow soluble reaction product.

RESULTS

Sephacryl S-300 Column Chromatography and Bioassays:

Column chromatography of concentrated
carotovoricin from non-induced cultures of *Ecc*
strain 379 after 52 h at 20 C showed that the total
protein was distributed between two peaks (Fig.
1a). Unlike the situation in induced cultures
(Chapter 1), the majority of the protein was found
in the lower molecular weight fraction. Bioassays
of these two peaks showed that the large molecular
weight fraction (peak 1) produced clear plaques on
indicators, while the small molecular weight
fraction (peak 2) produced diffuse plaques (Fig.
2b).

Ecc strain 379 showed a temperature-sensitive
resistance to erythromycin (30 ug/ml) and
chloramphenicol (15 ug/ml). Resistance to these
antibiotics was expressed at 20 C in LB but was
lost at temperatures above 35 C. *Ecc* strain 379
was also able to grow and pit CVP after 24 h at 24
C or 37 C. Mitomycin induced cultures of *Ecc*
strain 379 grown at 37 C produced no large
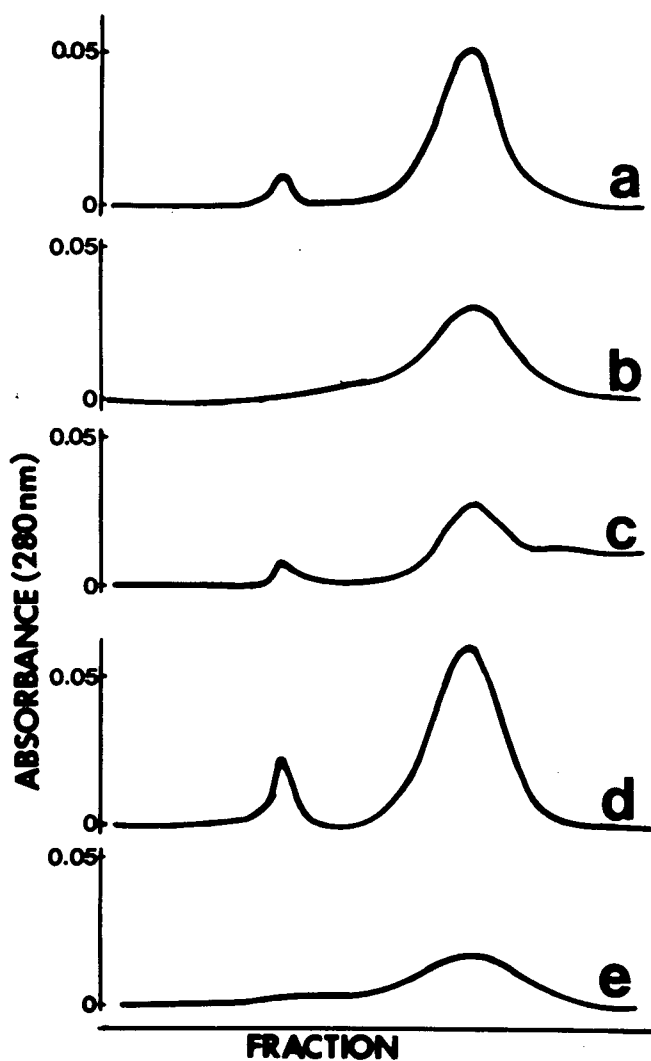
Figure 1. Sephacryl S-300 column chromatograms of carotovoricin from *Erwinia* and *E. coli* transconjugants.

- a. *Erwinia carotovora* subsp. *carotovora* (*Ecc*) strain 379 non-induced;
- b. *Ecc* strain 379 + mitomycin (0.2 ug/ml) at 37 C;
- c. R68.45 mediated CVP⁻ *E. coli* transconjugant;
- d. R68.45 mediated CVP⁺ *E. coli* transconjugant;
- e. Wild-type mated CVP⁻ *E. coli* transconjugates.

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Figure 2. Carotovoricin plate assays of colonies
and Sephacryl S-300 fractionated peaks from
concentrated supernatants of *E. coli* transcon-
jugants and *E. carotovora* subsp. *carotovora* (*Ecc*)
strain 379.

  a. Carotovoricin plate assays of colonies and
     fractionated large (peak 1) and small (peak 2)
     molecular weight bacteriocin components from
     supernatants of CVP⁺ and CVP⁻ *E. coli*
     transconjugants.

           CVP⁻   CVP⁺   CVP⁻
          colony   peak 1   colony

           CVP⁺   CVP⁻   CVP⁺
          peak 2   colony   peak 1

           CVP⁺   CVP⁺   CVP⁺
          colony   peak 2   colony

  b. Carotovoricin plate assays of colonies and
     separated large and small molecular weight
     bacteriocin components from supernatants of
     Ecc 379.

           peak 2   peak 1   peak 2

           peak 2   producing   producing
                         colony   colony

a

b
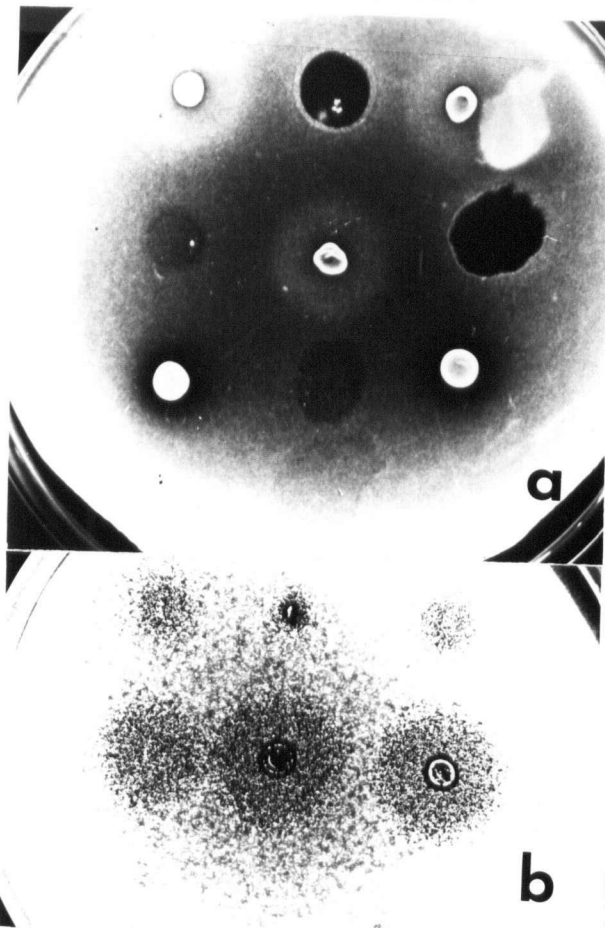
molecular weight carotovoricin as detected by
bioassay or S-300 column chromatography (Fig. 1b).

Column chromatography of concentrated
carotovoricin from mitomycin-induced cultures of
Ecc strain 379 grown at 37 C showed only one peak
corresponding to the low molecular weight component
(Fig. 1b). Bioassays of this peak produced
diffuse-type plaques on all indicators except 530
which was resistant. In addition, *Ecc* strain 379
grown at 37 C showed no visible signs of cells
lysis or reduction in cell turbidity.

Column chromatography of supernatants of R68.45
mediated CVP⁻ *E. coli* transconjugants showed two
peaks (Fig. 1c). However only the low molecular
weight component had diffuse-type carotovoricin
activity. Chromatography of concentrated
bacteriocin from wild-type mated CVP⁻, *E. coli*
transconjugants also showed one small molecular
weight bioactive component (Fig. 1e). In contrast,
column chromatography of R68.45 mediated CVP⁺, *E.
coli* gave a peak profile similar to that of non-
induced *Ecc* strain 379 (Fig. 1d) and produced
carotovoricin similar to *Ecc* 379 (Fig. 2a).

The ability to grow on CVP was transferred at a

frequency of 1×10^{-5}. Resistance to erythromycin (ery^r) (30 ug/ml) and chloramphenicol (cam^r) (15 ug/ml) was transferred at a frequency of 1×10^{-3}. When the erythromycin and chloramphenicol resistant transconjugants were tested for growth on CVP and production of carotovoricin, most were CVP^- and produced diffuse-type plaques on all indicators except strain 530 which was resistant. However 1.7% were CVP^+ and produced wild-type bacteriocin against all four indicators.

Transconjugants from wild-type matings were obtained at a frequency of 10^{-4}. When these were bioassayed for bacteriocin production, they produced diffuse-type plaques on three of the four indicators. All of these transconjugants were CVP^- and resistant to erythromycin (30 ug/ml) and chloramphenicol (15 ug/ml). In all transconjugants, alkaline phosphatase which is normally restricted to the periplasm of *E. coli*, was found in the filter-sterilized supernatants. No alkaline phosphatase activity was found in the supernatants of wild-type *E. coli*.

DNA Content:

Plasmid profiles of CVP$^-$, ery^r, cam^r, kanamycin
resistant (kan^r) transconjugants showed that they
contained both R68.45 and a second larger molecular
weight plasmid found in wild-type *Ecc* strain 379.
These plasmids existed in the transconjugant *E.
coli* as separate entities and R68.45 in these
transconjugants contained no insert (Fig. 3a).

Plasmid profiles of transconjugants obtained in
wild-type matings illustrated the transfer of the
large *Erwinia* plasmid (Erwp) (Fig. 3b) along with
the phenotypes of erythromycin resistance,
chloramphenicol resistance and diffuse-type
bacteriocin production. Unlike the *Erwinia* donors,
expression of the resistant phenotypes was not
affected at 37 C in the *E. coli* transconjugants.

Electron Microscopy:

Electron micrographs of supernatants of
transconjugant *E. coli* showed numerous membrane-
bound vesicles. Electron microscopy of intact CVP$^-$
transconjugants showed a protuberance of many
surface vesicles or "blebs" (Fig. 4a) which were
absent in non-transconjugant *E. coli*.
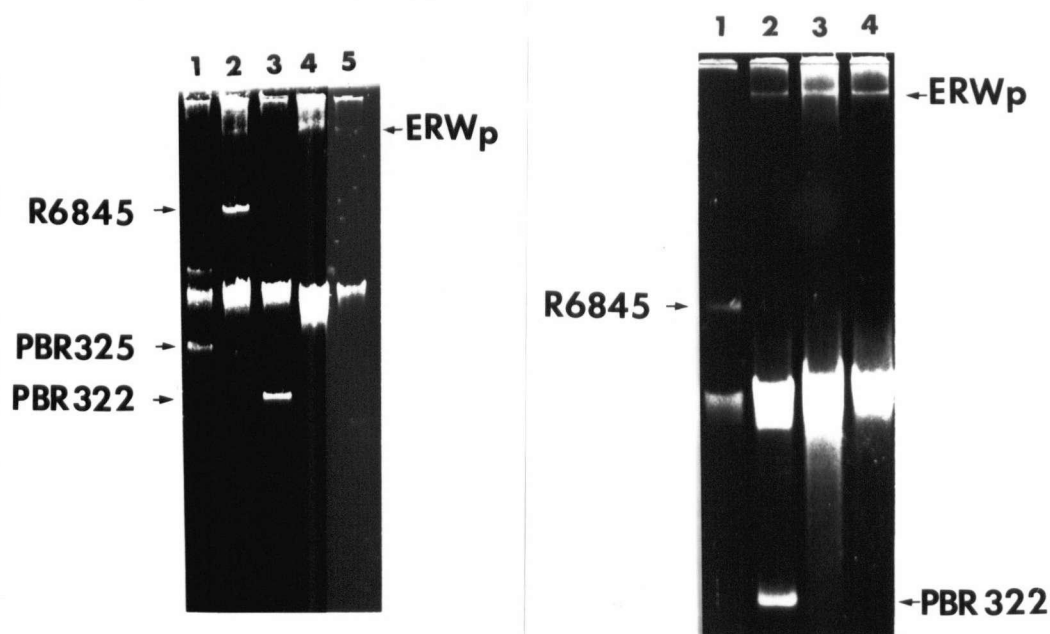
Figure 3. Total DNA analysis of wild-type *E. coli*, wild-type *E. carotovora* subsp. *carotovora* strain 379 (*Ecc* 379) and *Ecc* 379 x *E. coli* transconjugants with and without R68.45 mediation. Samples were run on a 0.5% agarose gel using Tris-Borate EDTA (TBE) at 1 to 1.5 V/cm for 18-24 h.

    a. R68.45 mediated *E. coli* transconjugants:
       Lanes:
       1) *E. coli* with pBR325;
       2) R68.45 mediated CVP⁻ *E. coli*
          transconjugant;
       3) *E. coli* with pBR322;
       4) *Ecc* strain 379 with *Erwinia* plasmid (Ewrp)
          10 ul sample load;
       5) *Ecc* strain 379 with Ewrp 5 ul sample load;

    b. Wild-type mated *E. coli* transconjugants:
       Lanes:
       1) HB101 with R68.45
       2) *E. coli* transconjugant with pBR322 and
          Erwp;
       3) *Ecc* 379 with Erwp;
       4) HB101 transconjugant with Erwp.
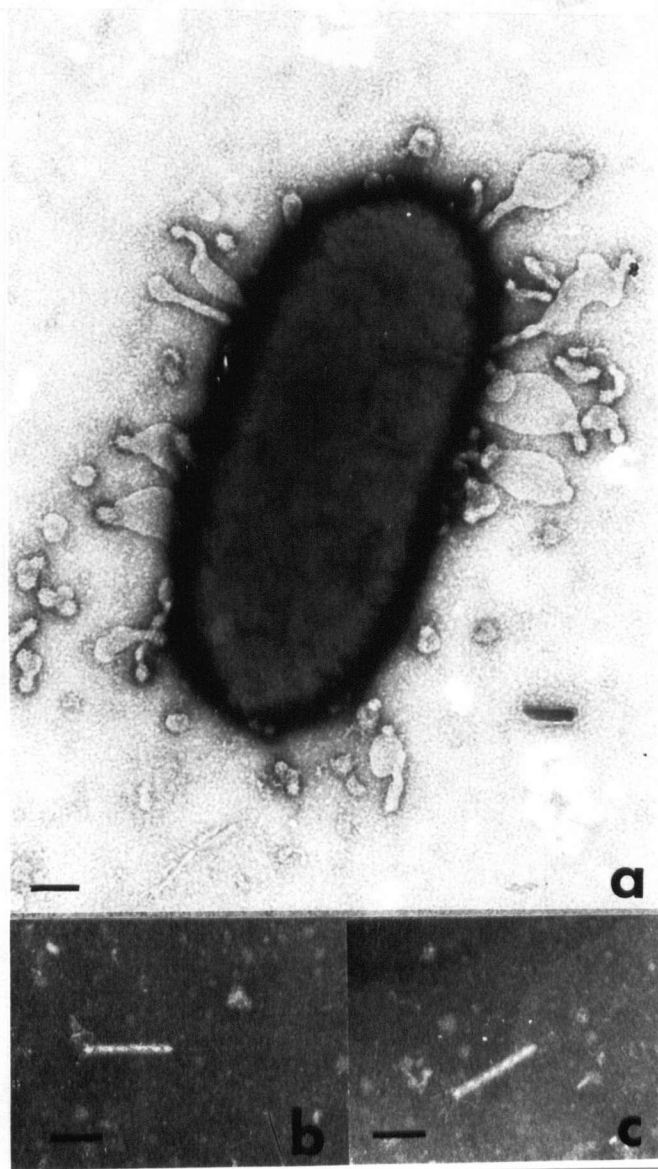
Figure 4. Electron micrographs of cells and
supernatants of *E. coli* transconjugants
(Bar=100nm).
  a. Typical negative stained *E. coli*
     transconjugant cell showing surface vesicles;
b,c. Supernatants of CVP+ *E. coli*
     transconjugants showing particulate
     carotovoricin. Note presence of tail fibres
     in c.

Electron micrographs of the large molecular weight fraction from CVP⁺ *E. coli* transconjugants showed particulate bacteriocins identical to those isolated from *Ecc* preparations (Fig. 4b-c).

DISCUSSION

Genetic analysis of carotovoricin production in *Erwinia* proved to be a substantial problem as convenient, wild-type markers were not available. However, tests used in the taxonomy of *Erwinia* species have consistently used resistance to erythromycin to separate *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* from other *Erwinia* (Schaad 1980).

Erythromycin resistance (30 ug/ml) in *Ecc* strain 379 was shown to be temperature sensitive with a loss of this phenotype at 37 C. When several other antibiotics were tested on *Ecc* strain 379, a chloramphenicol resistant (15 ug/ml) phenotype was found which, like erythromycin resistance, was also temperature sensitive. Cell

lysis, as seen by a reduction in turbidity of
induced cells was also inhibited at 37 C, as was
production of particulate bacteriocin. The
temperature sensitivity of the phenotypes suggested
a possible plasmid involvement due to the general
curing properties of growth at elevated
temperatures (Brock 1979).

In order to distinguish between chromosomal
and/or plasmid involvement in these temperature
sensitive phenotypes, transfer of genetic
information from *Ecc* strain 379 to a model system,
namely *E. coli*, was necessary. The conjugative
mobilization vector R68.45 was chosen because of
its wide host range, chromosome mobilization
ability (cma$^+$) from many origins, (Haas and
Holloway 1976) and ability to mobilize resident
plasmids at high frequency (Willetts and Crowther
1980). The mobilization ability of R68.45 is
thought to occur via a co-integration and
subsequent resolution in the recipient cell. The
resolution of the co-integrate is thought to be
recA dependent (Puhler and Riess 1984) with co-
integrate maintenance in recA minus recipients.
Furthermore, R68.45 contains the antibiotic markers

for kanamycin, tetracycline, and ampicillin (or carbenicillin) resistance which make it convenient to follow this plasmid through a population.

The ability to grow on CVP was transferred to *E. coli* at a frequency of 10^{-5} per recipient of R68.45. All CVP$^+$ transconjugants produced particulate bacteriocin. These facts suggested chromosomally derived determinants. Erythromycin (30 ug/ml) and chloramphenicol (15 ug/ml) resistances were transferred at a frequency of 10^{-3} per recipient cell. This high frequency suggested plasmid derived determinants. DNA analysis by agarose gel electrophoresis of CVP$^+$ ery^r, cam^r transconjugants showed the presence of a plasmid which corresponded to that found in wild-type *Erwinia*. However, these transconjugants were also kan^r and bacteriocin positive. These phenotypes were presumably transferred as an R68.45 co-integrate from the *Erwinia* chromosome. The size of such a co-integrate would be too large to resolve intact electrophoretically.

DNA analysis by AGE of CVP$^-$, ery^r, cam^r, kan^r transconjugants confirmed the presence of two plasmids. One of the plasmids was identified by

size as R68.45 with no insert and the other corresponded to a large molecular weight plasmid found in wild-type *Ecc* strain 379. Because the recipient cell (*E. coli* HB101) was a recA⁻ strain, visualization of separate plasmids suggested that the *Erwinia* plasmid transferred independently of R68.45. Bioassays using these transconjugants showed that they produced small molecular weight diffuse-type plaques. This bacteriocin behaved identically to that obtained from *Ecc* induced at 37 C or the small molecular weight component of carotovoricin-379 produced at 20 C.

Transconjugants obtained in wild-type matings between *Ecc* and *E. coli* confirmed the self-transmissibility of the large molecular weight plasmid in *Ecc* 379. All of these transconjugants were also CVP⁻, ery^r, cam^r and produced a small molecular weight bacteriocin component identical to that produced by the CVP⁻ R68.45 mediated transconjugants. From these results we conclude that this self-transmissible *Erwinia* megaplasmid coded for erythromycin and chloramphenicol resistance and a small molecular weight bacteriocin component. The production in *E. coli*

transconjugants of particulate bacteriocin
identical to that of *Ecc* 379 was only detected when
chromosomal constituents were transferred along
with the megaplasmid. This fact suggests that
carotovoricin-379 production in *Ecc* strain 379 is
coded for by both chromosomal and plasmid
constituents.

Electron microscopy of all transconjugants
showed that they produced many surface vesicles or
blebs not found in wild-type *E. coli*. Blebs have
been associated with endotoxin secretion in
Neisseria meningitidis (Devoe and Gilchrist 1973),
exo-enzyme production in *Pseudomonas aeruginosa*
(Thompson *et al.* 1985) and release of alkaline
phosphatase in *Pseudomonas* (Ingram and Dainty
1973). The presence of blebs on transconjugants
and wild-type *Erwinia* (Chapter 1), introduces the
possibility that this megaplasmid is involved in
the secretion of periplasmic proteins. This
suggestion is supported by the fact that in the *E.
coli* transconjugants, alkaline phosphatase which is
normally found in the periplasm, was found in
filter-sterilized supernatants. It would also
explain why in cosmid and shot-gun cloning

experiments with *Erwinia*, pectin-degrading enzymes
were found to accumulate in the periplasm of *E.
coli* transfectants or transformants and were not
effectively transported out into the supernatant
(Collmer *et al.* 1985; Zink and Chatterjee 1985;
Kotoujansky *et al.* 1985). These methods did not
take into account the possible involvement of a
resident megaplasmid in the secretion of
periplasmic proteins in *Erwinia*.

CHAPTER 3

SEROLOGICAL RELATIONSHIPS AMONG THE DIFFERENT

FORMS OF *ERWINIA* BACTERIOCIN DETECTED BY

POLYCLONAL ANTISERUM AGAINST PARTICULATE

CAROTOVORICIN-379

INTRODUCTION

Particulate bacteriocin produced by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) strain 379 bears a striking resemblance to the R-type pyocins of *Pseudomonas*. However, previous work (Chapter 1 and 2) has shown that carotovoricin-379 activity unlike pyocin activity can be divided into several fractions based on molecular weight. Production of the large molecular weight (particulate) fraction, which produced a clear-type plaque on several indicators, was temperature sensitive whereas the smaller molecular weight fractions were temperature independent and produced sparse or diffuse plaques. The addition of an outer membrane stabilizing agent resulted in the detection of total carotovoricin

activity at 20 C in one large molecular weight peak
which contained particles resembling pyocins and
when bioassayed, produced clear-type plaques.

The R-type pyocins of *Pseudomonas aeruginosa*
have structures which are closely related both
morphologically and serologically to bacteriophage
tails (Oshumi *et al.* 1980). They have been
considered model systems for particulate
bacteriocins. The distinguishing characteristic of
pyocins lies in their different activity spectra
against a range of indicator strains. This has led
to a division of the R-type pyocins into five
specificity groups (Shinomiya *et al.* 1979).
Analysis of the protein subunits of these pyocins
showed an almost identical composition with small
differences noted in the tail fiber region (Oshumi
et al. 1980). These small differences were
detected in cross-adsorption studies using specific
antisera and has led to the conclusion that these
fibers are a major determinant in the activity
spectrum of a given pyocin (Oshumi *et al.* 1980).
The genetic determinants for pyocin production are
thought to be located on the bacterial chromosome
and in most cases pyocin production is not a

temperature sensitive trait.

Genetic transfer from an *Erwinia* carotovoricin producer (*Ecc* strain 379) to *Escherichia coli* showed that a large molecular weight self-transmissible plasmid in *Ecc* strain 379 coded for erythromycin and chloramphenicol resistance, the production of a small molecular weight bacteriocin component and the proliferation of surface vesicles or blebs (Chapter 2). The small molecular weight carotovoricin component produced by *E. coli* transconjugants had the same activity spectrum as the small molecular weight fraction isolated from non-induced cultures of wild-type *Ecc* strain 379 grown at 20 C or induced *Ecc* 379 grown at 37 C. The purpose of this study was to develop antiserum to the large molecular weight fraction of carotovoricin-379 and to determine the relatedness of the mature intact particulate carotovoricin-379 and the particulate and soluble forms of carotovoricin produced by wild-type *Ecc* 379 and several *Ecc* x *E. coli* transconjugants.

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MATERIALS AND METHODS

Bacterial strains

Bacterial strains used and transconjugants were obtained as outlined in Chapters 1 and 2. Media and growth conditions were described in Chapters 1 and 2. Concentration and fractionation of carotovoricin were performed as outlined in Chapter 1.

Development of Antiserum

Carotovoricin-379 was concentrated as previously described from *Ecc* strain 379 grown in minimal M9 media at 20 C, 48 h after induction. Concentrated protein from 100 ml of culture supernatant was analysed by Sephacryl S-300 column chromatography as previously described (Chapter 1 and 2). The large molecular weight peak was pooled and bioassayed to confirm activity. Ten milligrams of protein was mixed and emulsified with an equal volume of Freunds complete adjuvant (Sigma). This was injected intramuscularly into the hind leg of a rabbit, once a week, for 5 consecutive weeks. On the 6th week, 20 ml of blood, collected from the

ear vein, was allowed to clot at room temperature
for 1 hour (h) followed by 12 h (overnight) at 4 C.
The remaining serum was clarified by low speed
centrifugation (8000 x g) for 25 minutes (min).
Clarified serum was stored in 1 ml aliquots at -20
C.

Radial Immunodiffusion

Clarified serum was diluted 1/100 in 0.5% noble
agar (Difco) in 50mM sodium phosphate buffer pH 7.4
+ 0.85% NaCl (PBS) and poured into petri dishes to
a depth of 0.3 cm. Twenty ul of culture
supernatant were added to pre-cut wells and the
plates incubated for 24-48 h at 25 C.

Immuno-sensitive Electron Microscopy (ISEM)

Polyclonal antiserum was used to "trap"
bacteriocin particles on EM grids (Hill 1984) by
sequentially floating carbon colloidion grids on
one drop of the following solutions for the
indicated times: a) 1/2000 dilution of antiserum
in PBS for 15 min at 22 C; b) PBS pH 7.4 for 10 min
at 22 C; and c) filter-sterilized culture fluid for
15 min at 22 C. Grids were stained with 10-12

drops of 2% phosphotungstic acid (PTA) pH 7.0 and viewed on a Phillips EM-300 electron microscope.

Subsequent decoration of trapped particles (Hill 1984) was accomplished by further incubations on PBS pH 7.4 for 10 min at 22 C followed by 1/1000 dilution of antiserum in PBS for 15 min at 22 C. Grids were stained and viewed as above.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was carried out in 0.5 mm 8.0% acrylamide using Tris-glycine-SDS, essentially following the discontinuous system outlined by Laemmili (Laemmili 1970; Laemmili and Farre 1973). Concentrated supernatants were dissolved in loading buffer (100mM Tris-HCl pH 6.8 with 2% SDS and 5% beta-mercaptoethanol), boiled for 2 min and electrophoresed at 18 mA for 1.5 h using a mini-vertical slab gel unit (Hoefer Scientific SE-200). Gels were stained either for protein or used for Western blots (electroblotting).

Protein detection was accomplished by staining gels with 0.5% Coomassie brilliant blue R-250 in 45% methanol, 10% acetic acid (v/v) for 1 h

followed by destaining in 20% methanol, 10% acetic

acid (v/v). Gels were preserved by drying at 65 C

on a homemade slab-gel dryer. Gels for blotting

were soaked in transfer buffer (25mM Tris, 192mM

Glycine, 20% Methanol; pH 8.3) for 1 h followed by

electroblotting and immuno-detection of proteins.

Protein standards (Sigma) ranged from 30-200

kilodaltons.

Western Blotting

The transfer of proteins to nitrocellulose

(Western Blotting) (Towbin *et al.* 1979; Gershoni

and Palade, 1983) was accomplished at 40 V, 150 mA

for 12 h in a homemade blotting apparatus with

stainless steel rod electrodes. After blotting,

nitrocellulose with transferred proteins was washed

thrice for 15 min each in 20mM Tris 500mM NaCl, pH

7.5 (TBS). Protein binding sites were blocked with

a mixture of 2% gelatin and bovine serum albumin

(BSA) (Sigma) (1 mg/ml) in TBS for 2 h at 37 C.

The blot was washed twice for 15 min each in TBS

and incubated in a 1/1000 dilution of polyclonal

antiserum + 0.1% BSA in TBS for 2 h at 37 C. This

was followed by two 15-minute washes in TBS and

incubation in a 1/1500 dilution of goat anti-rabbit
IgG-horseradish peroxidase conjugate (Sigma) in TBS
+ 0.1% BSA for 2 h at 37 C. The blot was washed
twice for 15 min each in TBS and incubated for 5-15
min in substrate buffer (50mM Tris-HCl pH 7.5)
containing 1 mg/ml diaminobenzidine (DAB) + 1%
hydrogen peroxide (added immediately before use).
The reaction was stopped by rapid dilution of
substrate in TBS. Blots were dried and stored
between paper towels.

RESULTS

Antiserum against the large molecular weight
fraction (particulate carotovoricin-379) reacted
with the homologous antigen and produced several
circular precipitin lines in radial immunodiffusion
(RID) (Fig. 1). These patterns were identical
regardless of the growth medium used (LB or minimal
medium). Non-induced cultures of *Ecc* strain 379
also produced bacteriocin which reacted in RID with
antiserum against particulate carotovoricin-379
(Fig. 1). CVP⁺ *E. coli* transconjugants

379 induced (M9)

CVP⁻ wild-type
transconjugant

379 induced (M9)

379 induced (LB)

E. coli HB101
control

379 non-
induced (LB)

1/5 dilution
of 379 induced

CVP⁺ *E. coli*
transconjugant

379 non-induced

x spill

CVP⁻ R68.45
mediated
transconjugant

E. coli
R68.45 control
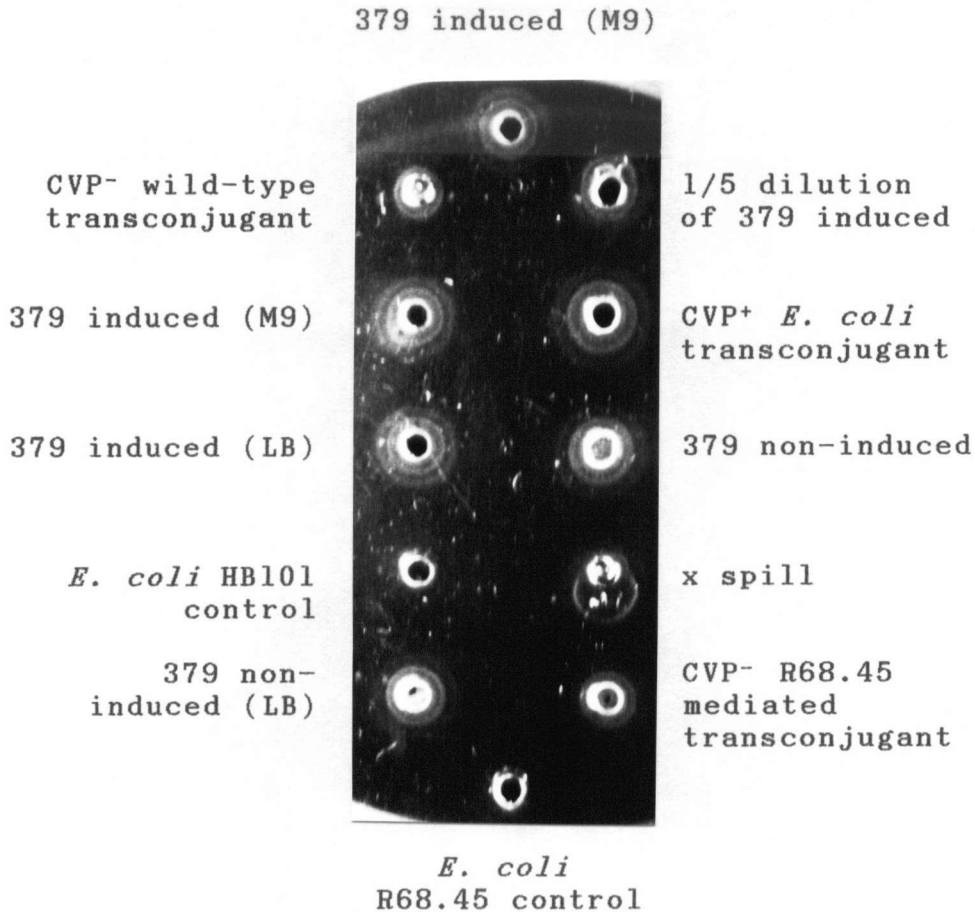
Figure 1. Radial immunodiffusion analysis of the relationship between particulate carotovoricin-379 produced by *Erwinia carotovora* subsp. *carotovora* strain 379 (*Ecc* 379) and *Ecc* 379 x *E. coli* trans-conjugants. Agar containing a final dilution of 1/100 of polyclonal antiserum against particulate carotovoricin-379 was poured. Cut wells were filled with supernatants from *Ecc* 379, *Escherichia coli* and *E. coli* transconjugants.

constitutively produced bacteriocins with
precipitin patterns similar to those of induced
cultures of *Ecc* strain 379. All CVP⁻
transconjugants produced only one major precipitin
band (Fig.1).

Immuno-sensitive Electron Microscopy (ISEM)

Antiserum directed against particulate
bacteriocin "trapped" a significantly higher number
of particles from 5 ul of an induced culture
supernatant (Fig. 2a and 2b). Trapped particles
included: intact carotovoricin with and without
attached heads, empty sheaths, cores and heads. In
addition, partially formed or disrupted bacteriocin
particles were also trapped on the same grid (Fig.
2b arrow).

When trapped particles were exposed to
additional antiserum they became coated (Fig. 2c).
Although they resembled intact particulate
carotovoricin-379, their outlines appeared less
distinct (Fig. 2c). In most cases these decorated
particles appeared out of focus but close
examination ruled out this possibility. The
particles were in fact heavily coated with
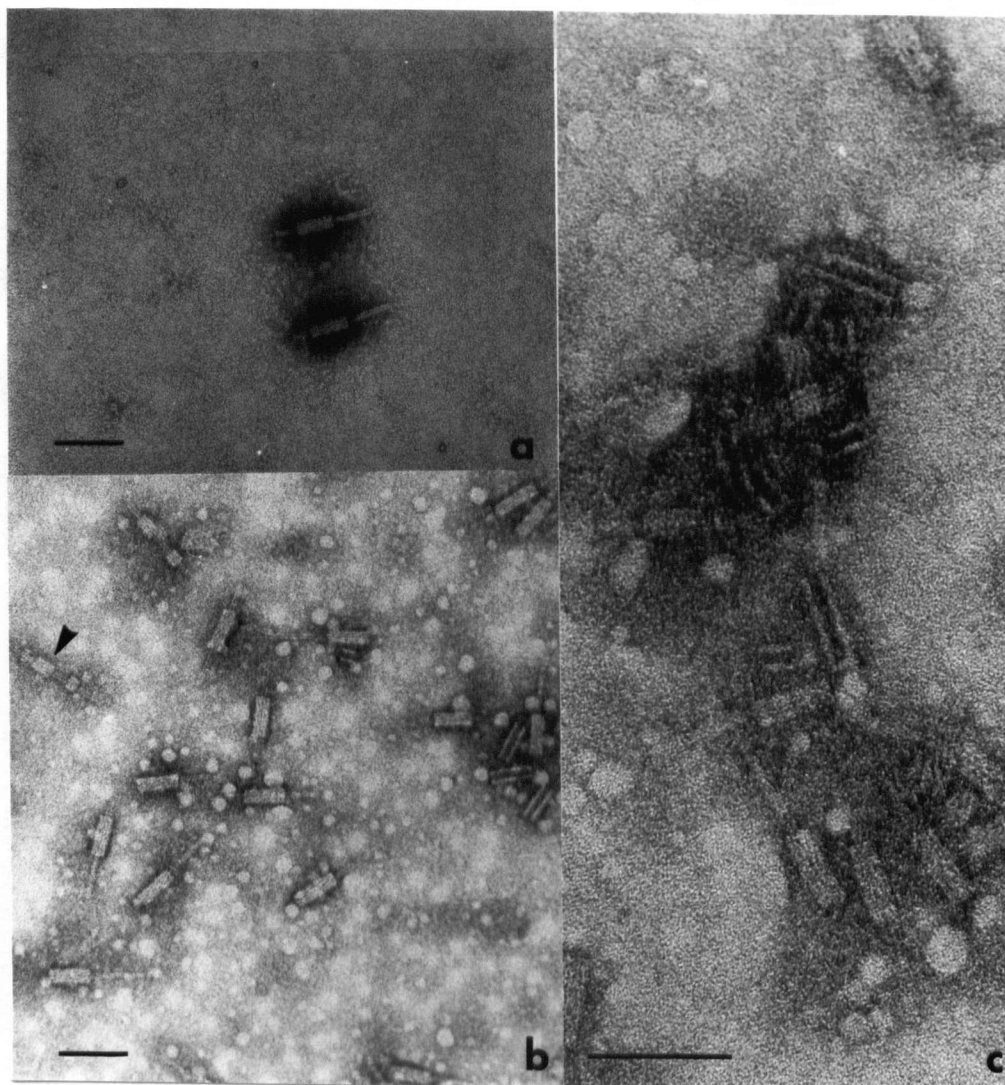
Figure 2. Detection of bacteriocins produced by
mitomycin C induced *Erwinia carotovora* subsp.
carotovora strain 379 by immunosensitive electron
microscopy employing polyclonal antiserum raised
against the large molecular weight peak obtained by
Sephacryl S-300 fractionation of supernatants
(Bars=100nm).
   a. Bacteriocin particles retained on uncoated
      grid receiving 5 ul of supernatant from an
      induced culture;
   b. 5 ul of supernatant from an induced culture
      applied to a grid pre-coated with polyclonal
      antiserum to bacteriocin (trapped bacter-
      iocin).   (Arrow shows partially disrupted
      particle).
   c. Trapped bacteriocin as in b, exposed to a
      second incubation with polyclonal antiserum
      (decoration).

antibodies and as a result were sometimes curved or slightly distorted (Fig. 2c).

SDS-PAGE

Polyacrylamide gel electrophoresis showed that the supernatant protein composition of *Ecc* 379 was complex and dependent on both the incubation temperature of the producing strain, and the incorporation of an inducing agent (Fig. 3a). At 20 C, induction with mitomycin C (0.2 ug/ml) resulted in a marked increase in several high and low molecular weight protein subunits in addition to a general increase in all protein subunits found in non-induced culture supernatants (Fig. 3a). Incubation at 37 C reduced supernatant protein concentrations by approximately 50-fold and abolished the induction mechanism active at 20 C (Fig. 3a).

All *E. coli* transconjugants showed a general increase in protein content in supernatants when compared with wild-type *E. coli* (Fig. 3b and 3c). CVP[+] *E. coli* transconjugants produced supernatant protein profiles indistinguishable from induced *Ecc* strain 379 (Fig. 3b and 3c). However, unlike *Ecc*

Figure 3. SDS-polyacrylamide gel electrophoresis of concentrated supernatants of *Erwinia carotovora* subsp. *carotovora* strain 379 and *Escherichia coli* transconjugants.

a. Effect of temperature and mitomycin C on production of supernatant proteins by *Ecc* 379:
   Lanes:
   1) Induced at 20 C;
   2,3) Peak 2 from Sephacryl S-300 fractionated non-induced culture at 20 C;
   4) Induced at 20 C;
   5,6) Non-induced at 20 C;
   7) Non-induced at 37 C;
   8) Induced at 37 C;
   9,10) Induced at 20 C.

b. Comparison of *Ecc* 379 with *E. coli* transconjugants:
   Lanes:
   1) Sigma standards (30 to 200 KD);
   2) R68.45 CVP⁻ transconjugants;
   3) CVP⁻ wild-type mated transconjugant;
   4) CVP⁺ R68.45 transconjugant;
   5) *Ecc* 379 induced at 20 C;
   6) *Ecc* 379 non-induced at 20 C;
   7) *E. coli* HB101 control;
   8,9) Sigma standards (30 to 200 KD).

c. Comparison of *Ecc* 379 with *E. coli* transconjugants:
   Lanes:
   1) Sigma standards (30 to 200 KD);
   2) *E. coli* HB101/R68.45 control;
   3) *Ecc* 379 non-induced at 20 C;
   4) *Ecc* 379 induced at 20 C;
   5) *E. coli* R68.45 CVP⁺ transconjugant;
   6) CVP⁻ R68.45 transconjugant;
   7) HB101 wild-type mated transconjugant CVP⁻;
   8,9) *Ecc* 379 at 37 C;
   10) Sigma standards (30 to 200 KD).
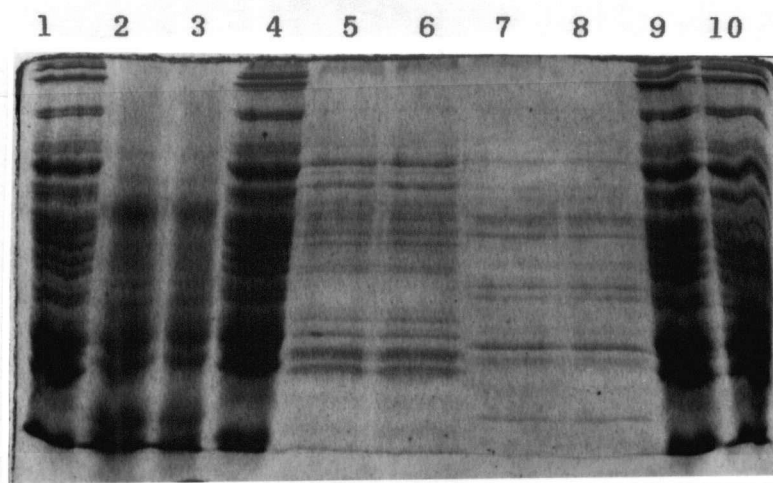
a.

b.

c.

strain 379, the proteins produced by CVP⁺ *E. coli*
were independent of both an inducing agent or
temperature. CVP⁻ *E. coli* transconjugants in
general, produced higher levels of extracellular
protein than wild-type *E. coli* HB101. A comparison
of proteins produced by CVP⁺ *E. coli* or induced
Erwinia, with CVP⁻ *E. coli* transconjugants showed
that several high and low molecular weight protein
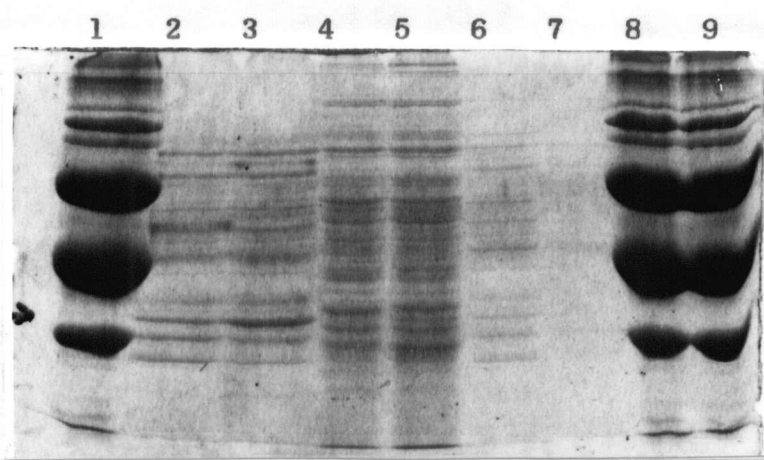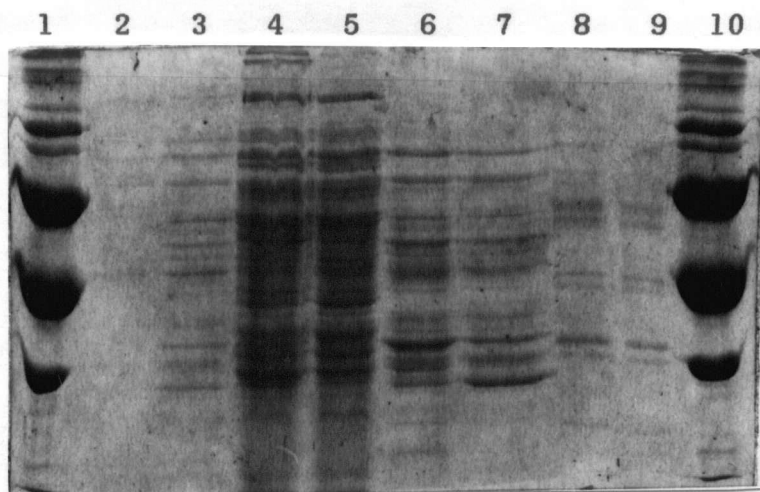subunits were not produced by CVP⁻ transconjugants
(Fig. 3b and 3c), and no particulate bacteriocin
was seen by EM.

Western Blotting

Most protein bands of the homologous antigen,
when blotted to nitrocellulose, reacted with the
antiserum against particulate carotovoricin-379
(Fig. 4). The most reactive subunits of the
homologous antigen were the high and low molecular
weight components (Fig. 4). These components were
in lower concentration in non-induced cultures and
absent from CVP⁻ transconjugants. A comparison of
immuno-reactive supernatant proteins from CVP⁺ E.
coli transconjugants with those of induced *Ecc*
illustrated some differences in the low molecular
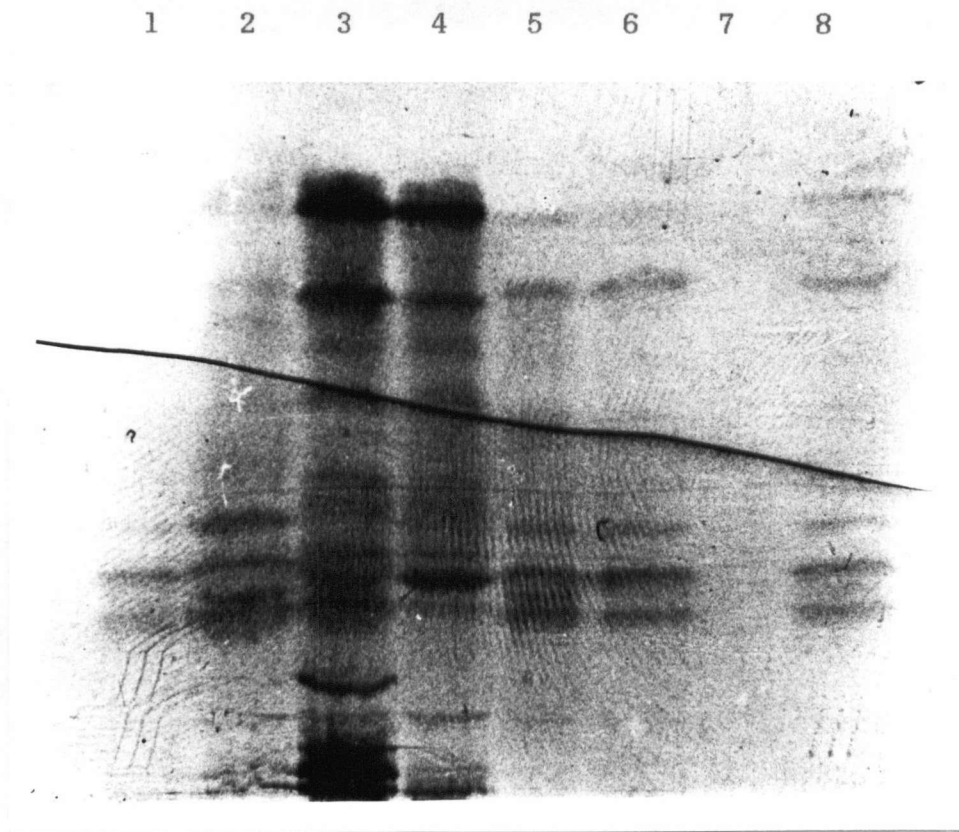
Figure 4. Western blot on nitrocellulose of supernatant proteins from *Erwinia carotovora* subsp. *carotovora* strain 379 and *Escherichia coli* trans-conjugants followed by immunodetection using polyclonal antiserum against particulate carotovoricin−379. Lanes:
1) Wild−type mated CVP⁻ *E. coli* transconjugant;
2) Non−induced *Ecc* 379 at 20 C;
3) Induced *Ecc* 379 at 20 C;
4) CVP⁺ R68.45 mediated *E. coli* transconjugant;
5) CVP⁻ R68.45 mediated *E. coli* transconjugant;
6) Wild−type mated CVP⁻ *E. coli* transconjugant with pBR322;
7) *E. coli* HB101 with R68.45 control;
8) CVP⁻ R68.45 mediated *E. coli* transconjugant with pBR322.

weight region, in spite of no apparent differences in bacteriocin activity or appearance (via EM or SDS-PAGE). CVP$^+$ transconjugants showed a relatively smaller percentage of immuno-reactive subunits (Fig. 4) when compared to the reactive proteins of *Ecc* (Fig. 3b and 3c).

DISCUSSION

The protein subunit composition of carotovoricin-379 as analysed by SDS-PAGE and Western blotting illustrated a complex structure consisting of over 20 subunits with a wide range of molecular weights. The protein composition of supernatants of CVP$^+$ *E. coli* transconjugants as seen by SDS-PAGE was identical to wild-type *Erwinia*. These CVP$^+$ transconjugants produced bacteriocin at 37 C without an inducing agent (Fig. 3c and 4). In contrast, an inducing agent dramatically increased the bacteriocin titre at 20 C in *Erwinia* but had no effect at 37 C (Fig. 4). CVP$^-$ transconjugants released proteins, some of which corresponded to bands present in wild-type

carotovoricin-379. The most obvious difference was the absence of high and low molecular weight protein subunits.

Immuno-detection of blotted proteins by antiserum against particulate bacteriocin demonstrated that particulate bacteriocin produced by CVP⁺ *E. coli* was very closely related to wild-type carotovoricin-379 produced by *Ecc* strain 379. However, small differences were noted in the low molecular weight region. The most obvious difference was the presence in the *Ecc* bacteriocin of a strongly immuno-reactive band corresponding to about 15,000 MW. This is most likely due to an outer membrane component of the carotovoricin-379 and the antigenic differences in the outer membrane components of the *Ecc* and *E. coli* producers. In addition, some protein subunits were detected with greater sensitivity. This was most likely due to their different immunogenic properties and positions in the intact particulate bacteriocin. CVP⁻ transconjugants showed three immuno-reactive small molecular weight subunits and one large subunit which was also present in CVP⁺ transconjugants. CVP⁺ transconjugants contained an

additional large molecular weight (about 150 kd) protein and several very low molecular weight components (less than 20,000) not present in CVP⁻ transconjugants. A comparison of protein stained bands with immuno-stained bands showed that a large number of protein subunits did not stain immunogenically in CVP⁻ transconjugants. This reduction in immunoreactive proteins does not seem to be as obvious in CVP⁺ transconjugants and may reflect a preference in a hypothetical secretion system for *Erwinia*-derived proteins. CVP⁻ transconjugants produced fewer *Erwinia*-derived proteins possibly allowing a less specific substitution of *E. coli* periplasmic proteins. This hypothesis was supported by the fact that CVP⁻ transconjugants produce surface vesicles and that alkaline phosphatase was found in the supernatant of these transconjugants (Chapter 2).

The polyclonal antiserum developed against particulate carotovoricin-379 consistently reacted with the homologous antigen. In addition, this antiserum also reacted with carotovoricin produced by all *E. coli* transconjugants. This fact suggests

that the low molecular weight carotovoricin is a
component of intact particulate carotovoricin.

GENERAL DISCUSSION

Ultrastructural examination of carotovoricin-
379 and carotovoricin-producing cells led to the
hypothesis of a carotovoricin release without lysis
(Chapter 1). Supporting evidence for this
hypothesis came from the fact that non-induced *Ecc*
strain 379 produced particulate bacteriocin
constitutively with no detectable reduction in cell
turbidity or viability. Examination of
carotovoricin-379 within the supernatant of non-
induced *Ecc* strain 379 showed that activity could
be fractionated into two components (Chapter 2).
The higher molecular weight component was much more
effective at inhibiting indicator strains than the
smaller molecular weight component (Chapters 1 and
2). Induction with DNA damaging agents skewed the
proportion of bacteriocin in the supernatant
towards the higher molecular weight component
(Chapter 1). A more careful examination of this
induction process illustrated a gradual stepwise
increase in the molecular weight of carotovoricin-
379 (Chapter 1).

Examination of induced producing cells indicated that a small proportion (about 10%) of these cells contained subunit-like surface projections at various stages of elongation (Chapter 1). Structural examination of carotovoricin-379 showed that the cellular projections resembled the central cores of particulate carotovoricin-379 (Chapter 1). In addition, the entire carotovoricin particle seemed to be arranged in a modular manner surrounding these central cores (Chapter 1). It was hypothesized that these cellular projections represented the central cores of carotovoricin and that intact particulate bacteriocin may be formed by an inducible addition of components to these cores resulting in an increase in molecular weight and bioactivity. In order to investigate this possibility, carotovoricin particles and cellular projections were examined by EM at several stages after induction (Chapter 1). The results showed that carotovoricin-379 particles gradually increased in size, molecular weight, and bioactivity after induction. In addition, cellular projections were observed which resembled these

different carotovoricin particles (Chapter 1).

An examination of fractionated carotovoricin-379 showed that the high molecular weight component contained intact particulate carotovoricin in both extended and contracted forms, whereas the low molecular weight component contained separated components of carotovoricin-379 (Chapter 1). Based on these observations, a hypothetical model for carotovoricin-379 production was developed in Chapter 1. The model outlined the construction of carotovoricin-379 at the level of the outer membrane by induced or non-induced cells.

In the model, carotovoricin-379 production was hypothesized as an inducible addition of protein subunits to a fimbrae-like projection. This projection formed the central core of carotovoricin-379 and swelled terminally in a vesicle or head. In the non-induced state partially active carotovoricin components accumulated in the periplasmic space and were secreted in membrane vesicles or blebs.

An interesting feature of particulate carotovoricin-379 production was that it is temperature sensitive (Chapter 2). Several other

phenotypes, including cell lysis following
induction and antibiotic resistance, were also
temperature sensitive in *Ecc* 379 (Chapter 2). This
fact introduced the possible involvement of plasmid
DNA in the production of carotovoricin-379.
Genetic transfer from *Ecc* to *E. coli* mediated by
R68.45 (Chapter 2) suggested that particulate
bacteriocin was coded for by chromosomal
constituents (Chapter 2). However, conjugation
studies without a mobilization vector showed that
Ecc 379 harboured a large molecular weight plasmid
which was self-transmissible, and coded for
erythromycin and chloramphenicol resistance and the
production of a low molecular weight bacteriocin
component (Chapter 2). This component corresponded
in activity and size to the partially active low
molecular weight component of carotovoricin-379.
Analysis of transconjugants by EM (Chapter 2)
showed a proliferation of surface vesicles or
"blebs". In addition, membrane vesicles and the
periplasmic enzyme alkaline phosphatase were found
in the supernatant of these cells (Chapter 2).

The production of membrane vesicles and
external alkaline phosphatase has been shown in

several pseudomonad species. Some are involved in
food spoilage and membrane vesicles have been shown
to increase the presence of a particular solid
substrate (Wing 1984). These vesicles have
recently been shown to be involved in enzyme
secretion (Thompson *et al.* 1985) Unlike most
bacteria, *Erwinia* produces a wide variety of
exoenzymes, some of which may be involved in
pathogenicity. The outer membrane of gram negative
bacteria provides a substantial barrier to release
of proteins (Nikaido 1985). Thus a secretory
system for macerating enzymes and possibly
carotovoricin components in *Erwinia* is necessary.

The formation of membrane vesicles in *E. coli*
transconjugants and the external release of
alkaline phosphatase (Chapter 2) along with a
general increase in proteins found in
transconjugant supernatants (Chapter 3) introduces
an additional involvement of the *Erwinia*
megaplasmid in secretion. In addition, the
production of a low molecular weight bacteriocin
component which corresponds in activity and plaque
morphology to the low molecular weight
carotovoricin components isolated in Chapter 1

suggests that carotovoricin-379 production may
involve both plasmid and chromosomal constituents.

The low molecular weight carotovoricin-379
component was shown in Chapter 1 to be dependent
upon induction and the incorporation of magnesium
in media. The model (Chapter 1) explained this
component as a prematurely released, partially
active bacteriocin component. The effect of
magnesium presumably stabilized outer membrane
vesicles inhibiting this premature release (Chapter
1). The representation in the model assumed that
the low molecular weight component was a subset of
components physically associated with intact
particulate carotovoricin-379. Support for this
came from the fact that polyclonal antiserum,
developed against particulate carotovoricin-379
reacted with the low molecular weight component
produced by CVP⁻ transconjugants (Chapter 3). One
possible explanation for this cross reaction may be
that the plasmid encoded low molecular weight
component could represent a group of proteins which
have some bacteriocin activity but are required for
the assembly of particulate carotovoricin-379.
This is not a novel explanation as K88 fimbrae in

E. coli have been shown to require a plasmid-encoded outer membrane protein for fimbral assembly. The absence of this polypeptide results in the accumulation of fimbral conponents in the periplasm (Hammond *et al.* 1984).

In order to speculate further on the nature of the low molecular weight component of *Ecc* 379, an examination of the mode of action of carotovoricin requires consideration. Itoh *et al.* (1980a) found that binding of particulate carotovoricin by indicators had at least two distinct direct effects. These two effects were cell lysis and metabolic death. In phospholipase A minus mutants, no detectable cell lysis was seen. The cells, however, were metabolically inactive (Itoh *et al.* 1981). This metabolic death was shown to be due to a loss in the energized state of the membrane which is used for ATP synthesis and nutrient uptake (Itoh *et al.* 1982). In earlier work by Itoh's group, it was shown that particulate carotovoricin contained an activator of membrane bound phospholipase. This activator was shown to cause cell lysis in wild-type indicators but not in phospholipase mutants. It was assumed that this phospholipase activation

was the primary mode of action of carotovoricin.

However, the phospholipase A mutants which bound

carotovoricin although intact were still

metabolically inactive (Itoh *et al.* 1982).

In view of the complex structure of

carotovoricin, it is quite conceivable that a

phospholipase activator may be coded for by the

self-transmissible plasmid observed in *Erwinia*.

This fact could result in a differing activity

spectra dependent upon the amount and type of

phospholipase present in indicator strains.

However, the primary mode of action of

carotovoricin is the inactivation of the energized

state of the membrane which may be due to the

formation of non-specific ion channels by the

central core of the particle. This state would

result in a degeneration of the proton motive force

(pmf) which results from metabolism. The constant

regeneration of this chemical and pH gradient

allows cells to couple thermodynamically favourable

tendencies to ATP synthesis and nutrient uptake.

Degeneration of the pmf would result in a cessation

of metabolism. This type of killing activity has

been reported using mutant T4 phage which contain

empty heads (T4 ghost) (Lewin 1977).

SUMMARY

1)   Several forms of carotovoricin-379 which
     differed in appearance, molecular weight and
     bioactivity existed in the supernatant of
     producing cells of *Erwinia carotovora* subsp.
     carotovora strain 379 (*Ecc* 379) grown in the
     absence of magnesium.

2)   The large molecular weight particulate form of
     carotovoricin-379, which gave a small clear
     zone of inhibition when bioassayed, resembled a
     bacteriophage tail, was temperature sens-
     itive and was inducible by mitomycin C.

3)   Production of the low molecular weight form of
     carotovoricin-379, which gave a diffuse zone of
     inhibition when bioassayed, was eliminated in
     media containing magnesium.

4)   Several cellular projections were also observed
     on producing cells. These projections may
     represent different stages of induced partic-

ulate carotovoricin construction on a fimbrae-like projection which swells to a detachable vesicular head.

5)   *Ecc* 379 showed temperature sensitive production of particulate carotovoricin-379, cell lysis after induction, and resistance to erythromycin and chloramphenicol.

6)   *Ecc* 379 contained a self-transmissible megaplasmid which coded for erythromycin and chloramphenicol resistance, a low molecular weight component of carotovoricin-379, and vesicle formation which possibly represents a secretory mechanism for *Erwinia* exoproteins.

7)   The small molecular weight carotovoricin-379 component coded for by the *Erwinia* plasmid is serologically related to particulate bacteriocin and contains protein subunits found in particulate bacteriocin.

8)   Particulate carotovoricin-379 production is coded for by both chromosomal and plasmid

determinants as wild-type particles were only
produced in *E. coli* when a chromosome mobil-
ization vector (R68.45) was used.

9) Carotovoricin production may be linked to
a plasmid-dependent secretory system for all
Erwinia exoproteins.

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