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Bacteriocins of Gram-Positive Bacteria

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INTRODUCTION

Until recent years most definitive investigations in the field of bacteriocins have centered on those of gram-negative bacteria and, in particular, on the prototype bacteriocins—the colicins. The rewards of this research effort, though selective, have undoubtedly been great. Sophisticated studies of several of the colicins have provided a wealth of detailed information pertaining to their physicochemical properties, mechanisms of action, and genetic determinants. Although earlier reviewers have kept pace with developments in the investigations of these bacteriocins (29, 80, 81, 112, 194, 233), reports of microbial antagonism associated with gram-positive bacteria have often been overlooked or understressed. In view of the steadily increasing number of publications dealing with bacteriocin-like antagonism produced by gram-positive organisms, it seems an opportune time to correct this imbalance. We therefore present an overview of the accumulat-

ing information on the bacteriocins of gram-positive bacteria and discuss some of the important problems that confront investigators in this field. Where appropriate, selected references have been made to pertinent data on bacteriocins of gram-negative species.

HISTORICAL PERSPECTIVES AND EMERGING DEFINITIONS

Recorded observations of antagonistic interactions between different bacteria probably date from those of Pasteur and Joubert (220) who noted the inhibitory effect of common bacteria from urine on *Bacillus anthracis*. The early investigators were more concerned with the biological implications of microbial interactions than with the chemical characterization of the inhibitory agents. Many of these early studies, exploring the possibility of controlling diseases such as anthrax and diphtheria by use of nonpathogenic, antagonistic microorganisms, have been adequately reviewed by Florey and associates (72, 73). Although the nature of the inhibitory mechanisms or substances was obscure, it seems likely that many of the observed interactions were caused by substances that are now classified as bacteriocins.

In the original definition (145), the term bacteriocin referred to proteins of the colicin type, characterized by lethal biosynthesis, intraspecific activity, and adsorption to specific receptors. It is now apparent that antagonistic substances of this general nature are ubiquitous. Closer study, however, has indicated that relatively few of these substances, especially those produced by gram-positive bacteria, fit closely in the classical colicin mold. A wide variety of often poorly characterized substances produced by bacteria have been somewhat loosely referred to as "bacteriocins."

Inhibitory bacterial products include a wide range of substances: "classical" low-molecular-weight antibiotics, metabolic products, lytic agents, enzymes, bacteriocins, and defective bacteriophages. The divisions within this spectrum are rather ill defined and in practice have exhibited considerable overlap. A precise definition of the bacteriocins appears elusive and, as will be discussed, may in fact be futile. Classical criteria were originally based on the characteristics of colicins. These criteria have been used in varying combinations and applied with different degrees of consistency and proof in defining other bacteriocins. (i) A narrow inhibitory spectrum of activity centered about the homologous species; (ii) the presence of an essential, biologically active protein moiety; (iii) a bactericidal mode of action; (iv) attachment to specific cell receptors; (v) plasmid-borne genetic determinants of bacteriocin production and of host cell bacteriocin immunity; (vi) production by lethal biosynthesis (i.e., commitment of the bacterium to produce a bacteriocin will ultimately lead to cell death).

The above criteria are generally applicable to the prototype bacteriocins, the colicins; however, a number of the bacteriocins produced by gram-positive bacteria show discrepancies. Hamon and Peron (107) commented upon some of the atypical features that seemed characteristically associated with bacteriocins of gram-positive bacteria. These included a wider spectrum of activity against organisms of different species and a less-solid host cell immunity to the homologous bacteriocin.

In examining a new antagonistic bacterial substance, it is relatively easy to determine whether it meets the first three criteria. Criteria (ii) and (iii) have been generally applicable to well-characterized bacteriocins of gram-positive, as well as gram-negative, organisms. Documentation that criteria (iv) and (v) are satisfied requires more extensive analysis and exceptions have been noted. Data on these points are not available for many bacteriocins. Criterion (vi) has only been studied in relation to a limited number of bacteriocins and may be found to be associated only with the inducible bacteriocins.

At present there are two problems in the use of the term bacteriocin: first, there is no universally accepted definition for this group of substances; and second, many of the described inhibitory substances have not been characterized sufficiently to fulfill any classification.

Therefore, it is best to follow the practice of many investigators (17, 62, 104, 136, 137, 157, 189) in designating incompletely defined antagonistic substances as bacteriocin-like and reserving the term bacteriocin to be tentatively applied to those bacterial substances that have been shown to meet at least the criteria (ii) and (iii) listed above.

DETECTION OF BACTERIOCIN-LIKE ANTAGONISM

Examination of a sufficiently large number of strains (100 or more) of any one species is generally rewarded with some evidence of bacteriocin-like antagonism. The prevalence of such antagonism in various gram-positive species is recorded in Table 1. It is not possible to draw meaningful comparisons between figures reported in these studies since the apparent frequency of inhibition by these strains is dependent upon several variables. Within each species, production of bacteriocin-like antago-

TABLE 1. *Frequency of bacteriocin-like antagonism in gram-positive bacteria*

Producer organism	No. tested	Positive antagonism (%)	Method of testing[a]	Reference
Bacillus cereus	24	50	D	91
B. megaterium	200	46	S	141
B. megaterium	100	53	S	204
B. stearothermophilus	22	55	D	253
B. thuringiensis	24	42	D	91
B. thuringiensis	17	71	D	62
Clostridium botulinum	12	83	D	16
C. perfringens	35	14	D	291
C. perfringens	237	2	D	244
C. perfringens	33	12	D	186
C. sporogenes	25	36	D	17
Clostridium spp.	106	17	S	127
Corynebacterium diphtheriae	441	94	D	88
C. diphtheriae	108	2	D	283
C. fermentans	29	31	D	285
C. pseudodiphtheriae	134	17	D	285
Corynebacterium spp.	308	12	D	284
Corynebacterium spp.	383	14	D	196
Lactobacillus fermenti	121	21	D	63
Lactobacillus spp.	199	6	D	64
Lactobacillus spp.	100	7	D	300
Listeria monocytogenes	8	50	D	290
L. monocytogenes	51	49	D	106
Mycobacterium spp.	79	100	D	279
Staphylococcus aureus	270	6	D	150
S. aureus	200	2	D	84
S. aureus	2,035	1	S	202
S. aureus	65	38	D	48
S. aureus	100	7	S	12
S. aureus	2,000	4	D	71
S. aureus	1,065	5	D	129
S. aureus	270	8	D	180
S. aureus	623	37	S	219
S. epidermidis	478	6	D	71
S. epidermidis	387	9	D	129
Staphylococcus spp.	1,239	11	D	173
Staphylococcus spp.	71	16	D	296
Staphylococcus spp.	939	6	D	226
Streptococci, alpha-hemolytic	270	72	S	87
Streptococci, alpha-hemolytic	215	54	S	222
Streptococci, alpha-hemolytic	120	78	D	54
Streptococci, beta-hemolytic	61	28	S	255
Streptococci, beta-hemolytic	107	35	S	227
Streptococci, group A	130	3	D	276
Streptococci, group A	71	54	S	213
Streptococci, group A	1,568	95	S	164
Streptococci, group B	121	7	D	166
Streptococci, group B	135	2	D + S	270
Streptococci, group B	45	67	D	299
Streptococci, group D	77	99	D	297
Streptococci, group D	81	65	D	159
Streptococci, group D	99	51	D	35
Streptococci, group D	81	95	D	287
Streptococci, group D	108	62	S	224
Streptococci, Oral	13	46	D	160
Streptococcus mutans	130	75	D	104
S. pneumoniae	30	33	D	199

[a] D, Deferred antagonism; S, simultaneous antagonism.

nism may be related to a particular phage type (48) or to a certain serotype (213). Other significant factors include the basic method and conditions of testing, the number and innate susceptibility of the indicator organisms utilized, the criteria adopted, and more subjective factors such as individual interpretations of the experimental findings.

Methods of Demonstrating Antagonism

For screening purposes, the general test for antagonism is performed on solid media and involves the detection of inhibition of growth of an indicator (passive) strain caused by the test (active) culture. The two basic methods that are commonly used are referred to as the simulta-

neous (or direct) and the deferred antagonism procedures.

The simplest direct test and the one most widely used for the preliminary screening of large numbers of strains is the "spot-on-lawn" antagonism, based on the method devised by Gratia (93). Here, the test and indicator cultures are grown simultaneously and the demonstration of antagonism is dependent upon the release of a diffusible inhibitor early in the growth of the test culture. The density of the indicator lawn is an important determinant of the sensitivity of the method (172). The lawn is generally seeded before inoculation of the test strains (277) but, alternatively, may be sprayed onto the surface subsequent to inoculating the potential antagonists (99, 100, 129, 150, 153, 222). Variations of this procedure include the use of overlapping drops (12, 239) and also of wells cut into freshly seeded pour plate cultures and filled with agar containing the test organism (242). A modification with enhanced sensitivity useful for screening facultative anaerobes is the inoculation of the test strains by stabbing them into an agar culture plate that has been seeded on the surface with the indicator strains (12, 63, 84). This method is well suited to the examination of single colonies and has had widespread application in genetic studies.

Methods of deferred antagonism were largely developed by Fredericq (79) and later have been modified by others for use in bacteriocin-typing procedures (1, 274). In deferred antagonism, the test organism is grown on agar for a period of time. The bacteria are then killed by exposure to chloroform or heat, and an overlay of the indicator strain in melted agar is placed on the surface. Deferred antagonism procedures often prove to be more sensitive than simultaneous antagonism and permit the independent variation of the time and conditions of incubation of the test and indicator cultures. These features may be useful when testing bacteriocin production under different conditions of aeration or at temperatures not conducive to growth of the indicator strain. A potential disadvantage of the deferred method is that some inhibitory agents may be inactivated on exposure to the chloroform vapours. For example, it was found in one study (35) that a bacteriocin produced by *Streptococcus faecalis* subsp. *zymogenes* was sensitive to chloroform. The authors overcame the problem by omitting the chloroform step, overlayering the indicator culture with care not to disturb the test culture. A useful alternative for freely diffusing inhibitors is to seed the indicator culture on the reverse side of the medium (91, 125, 158, 272). An additional advantage of this procedure is the exclusion of nondiffusing bacteriophage activity from consideration.

It is important to test by both basic methods when screening strains for inhibitory activity and also to use a wide range of different media and growth conditions. The optimal conditions for growth of the test strain do not necessarily coincide with those giving maximal bacteriocin production. Moreover, the composition of the medium may indirectly affect the sensitivity of the indicator strain. Growth of certain strains of *S. mutans* and *S. salivarius* on sucrose-supplemented medium promotes the formation of an extracellular layer of polysaccharide that protects the normally susceptible organisms from a bacteriocin of *S. mutans* (237). The influence of the composition of the medium and the conditions of incubation upon the demonstration of microbial antagonism cannot be overemphasized.

Application of these screening procedures gives useful preliminary information for the identification of possible bacteriocinogenic organisms. These methods suffer from the limitation of not specifically demonstrating bactericidal activity. It is important to exclude (or to identify) other potential causes of inhibitory effects that are not differentiated by these procedures (see below).

Antagonism Unrelated to Bacteriocin Production

When specifically investigating bacteriocins it is mandatory to eliminate unrelated phenomena that may mimic bacteriocin activity in tests such as those described above. In many bacterial species the initial observations of bacteriocin-like antagonism came as a by-product of the investigation of collections of strains for lysogeny (17, 64, 104, 138, 263, 269). An important differential characteristic is that bacteriocins, unlike bacteriophage, do not carry the genetic determinants necessary for self-replication within susceptible organisms. Thus, only bacteriophages can be propagated on cultures of the indicator strains. A difference may also be demonstrated by the appearance of individual plaques when increasing dilutions of bacteriophage-containing supernatants are spotted onto indicator lawns. Dilution to extinction of bacteriocin preparations gives diminishing zones, but no plaque formation. A potential problem is posed by strains yielding both bacteriocin and bacteriophage. When the bacteriophage is present in higher concentration, identification of the bacteriocin may be dependent upon the use of indicators susceptible only to

the bacteriocin (124, 141). Alternatively, the reverse-side agar technique may be utilized to exclude contact of the indicator strains with the bacteriophage. Differences in other physicochemical properties of bacteriophage and bacteriocin can be useful in some cases. Hamon and Peron (110) have examined other methods of distinguishing virulent bacteriophage from bacteriocins, the most useful of which is the greater resistance of bacteriophage to trypsin treatment. Bacteriocins are not affected by large doses of ultraviolet irradiation, and many bacteriocins are extremely heat resistant. These properties provide additional means of differentiating bacteriocins from phages. Exceptions to these generalizations do occur.

By-products of metabolism that may be capable of bacteriocin-like antagonism on solid media include ammonia (238), lactic acid (288), free fatty acids (307), and hydrogen peroxide (126, 188, 268, 310). Production of hydrogen peroxide may be prevented either by anaerobic incubation or by incubation on medium supplemented with catalase (126, 188, 268) or peroxidase (126). Several investigators, in testing for bacteriocin production by beta-hemolytic streptococci (164, 172, 213, 227), apparently did not exclude the possibility that inhibitory levels of hydrogen peroxide released by the test strains may have been responsible for the observed antagonism. Polarographic procedures have been utilized to demonstrate that bactericidal concentrations of hydrogen peroxide may accumulate in liquid cultures of group A streptococci (188).

Bacteria produce a wide variety of different bacteriolytic enzymes and low-molecular-weight inhibitors, some of which have bacteriocin-like properties and may prove difficult to categorize. The widespread occurrence of such substances makes it imperative to avoid premature or indiscriminate use of the term bacteriocin. In the past, insufficient attention has been paid to the adequate characterization of agents causing bacteriocin-like inhibitory effects. Investigators have pursued the potential of such agents as epidemiological markers or as in vivo therapeutic agents without adequate knowledge of the nature of the observed antagonism.

Many of the "classical" antibiotics produced by bacteria have been shown to be peptide in nature (19, 73, 100) and some seem to have many properties in common with substances referred to as bacteriocins. For example, the antibiotic nisin, which is produced by certain strains of *S. lactis*, seems similar to the bacteriocin, streptococcin A-FF22, produced by a group A streptococcus (Tagg, unpublished observations). Both appear to be produced in cell-associated and extracellular forms. They have similar physicochemical properties, similar molecular weights, and both seem to be plasmid determined. Diplococcin (216) is another antibiotic that is difficult to distinguish from the bacteriocins.

Bacteriolytic enzymes are produced by many bacteria and may be confused with bacteriocin effects. Staphylococcal cultures have been found to produce a variety of bacteriolytic and autolytic enzymes including virolysin (229), lysostaphin (247), lysozyme (114), endo-β-*N*-acetylglucosaminidase (306), and phage-associated lysin (265). Moreover, preparations of the delta-hemolysin of *Staphylococcus aureus* have also been shown to have antibiotic activity (103, 119) and it has been remarked (103) that similarities in heat tolerance, trypsin sensitivity, and poor antigenicity exist between this substance and the bacteriocin produced by *S. faecalis* subsp. *zymogenes*.

An additional source of confusion and of conflicting reports by different investigators is the production of several kinds of inhibitory or bactericidal agents, under the same or different conditions, by a strain or strains of the same species. Moreover, at present it seems that many inhibitory substances defy precise classification into any one existing category. One investigator's bacteriocin may very well be another's defective phage, bacteriolytic enzyme, or classical antibiotic. Unless some firm guidelines can be established to unequivocally define the bacteriocins, continued use of the term may be unjustified.

NOMENCLATURE AND CLASSIFICATION OF BACTERIOCINS

The naming of bacteriocins has been haphazard, at times being based upon the generic (78, 127, 170, 279, 300) and at other times the species (88, 91, 104, 138, 202, 253) designation of the producer strains. In gram-positive bacteria, this lack of uniformity is evidenced by the alternate designations of bacteriocins of *Listeria monocytogenes* as either listeriocins (290) or monocins (105), those of *Corynebacterium diphtheriae* as corycins (170) or diphthericins (88), and those of *S. aureus* as either staphylococcins (78) or aureocins (202). Similarly, bacteriocins of the genus *Clostridium* have been termed clostocins (127) or clostridiocins (43) or, alternatively, have received individual species designations such as boticin (157), butyricin (43), and either perfringocin (43, 292) or welchicin (246). In many cases authors have added a ter-

minal "e" to the name of the bacteriocin; for example, staphylococcine (78), listeriocine (290), and corycine (170). This convention appears most often in European publications and has not been adopted in the present review. Included among the recommendations for the naming of antibiotics (118) has been the suggestion that reference be made, by preference, to the genus of the producing organism.

Because different bacteriocins may be produced by organisms belonging to a single species, additional designations are required for further differentiation. These are generally arbitrary, often being consecutive letters of the alphabet (124, 127, 150, 291). For precise specification of a particular bacteriocin it has been suggested (207, 232) that the trivial designation of the producing strain be included within the bacteriocin name. This recommendation has gained widespread acceptance by investigators of the colicins (e.g., colicin E1-K30 is a colicin of type E1 produced by *Escherichia coli* strain K30) and as the number of papers describing bacteriocins of other gram-negative and of gram-positive species is increasing, it seems worthy of general application. In our own studies we now refer to the bacteriocin produced by group A *Streptococcus* strain FF22 as streptococcin A-FF22. Where possible this same convention has been followed in the present review.

Often the production of different bacteriocins by strains of a particular species is first suspected from variations in their activity spectra and this criterion is most frequently used as the basis for the provisional subdivision of a group of related bacteriocins. For example, Fredericq (79) was able to distinguish 17 colicin types on the basis of cross-resistance tests, using a series of specific resistant mutants derived from a sensitive indicator strain. The latter method, which is based upon the specificity of bacteriocin receptors, although found useful to establish divisions within the monocins (108), clostocins (127), and staphylococcins (78, 100), has not always been applicable to bacteriocins of other gram-positive bacteria. One problem has been the difficulty in obtaining bacteriocin-resistant mutants of organisms such as *Lactobacillus fermenti* (63) and *B. megaterium* (205). In such cases it may be possible to subdivide groups of bacteriocins into several types on the basis of differences in their activity spectra on a diverse group of naturally occurring strains (84, 246) often in combination with other distinctive characteristics such as production kinetics and mode of action for the megacins (124) or sensitivity to proteolytic enzymes, heat, and chloroform for some of the streptococcal bacteriocins (35, 287).

Strains producing more than one bacteriocin pose another problem. A strain of *S. faecalis* subsp. *zymogenes* was shown to produce two bacteriocins differing in chloroform sensitivity and in activity spectrum (35). Similarly, megacins A and C may be produced by a single strain of *B. megaterium* (68, 124).

PRODUCTION OF BACTERIOCINS

The conditions of incubation of bacteriocinogenic organisms strongly influence the effective yield of active bacteriocin. For many strains ill-defined factors appear to affect the production of bacteriocins and the optimal conditions may need to be determined empirically.

Composition of the Growth Medium

In a number of instances involving gram-positive organisms significant production of bacteriocin-like substances has been demonstrated only on solid media (21, 84, 104, 124, 155, 173, 289, 303). Kelstrup and Gibbons (160) found that increasing the viscosity of liquid media, by addition of agar, dextran, glycerol, or starch, increased the yield of bacteriocins produced by streptococci isolated from the oral cavity of humans and rodents. In another study the yield of staphylococcin 1580 was shown to be 20 times greater in a semisolid medium than in the corresponding liquid medium (153).

Particular medium components may be found to be critical for the production of individual bacteriocins. Bacteriocin production by various strains of *S. mutans* was enhanced by the addition of 2% yeast extract to a basal Trypticase medium (236). Similarly, the formation of butyricin 7423 in a semidefined medium was found dependent upon the amount of casein hydrolysate added, up to a maximum of 5% (43). The formation of bacteriocins by several corynebacteria (197) and by a staphylococcus (174) was said to be dependent upon the amino acid content of the culture medium. Manganese ions were found necessary for megacin production in chemically defined media (5). Addition of 0.5% mannitol to brain heart infusion broth enhanced the yield of staphylococcin 462 but decreased that of staphylococcin 414 (101). Similarly, glucose increases the production of streptococcin A-FF22 (unpublished observations) but reduces that of streptococcin B-74628 (270). Others have shown that the addition of either glucose or mannitol enhanced the production of an inhibitor by phage type 71 staphylococci in tryptic soy digest broth (13).

Conditions of Incubation

Variation of some culture conditions such as temperature, time, aeration, and pH has been shown to have profound effects on the yield of active bacteriocin.

Generally the production of bacteriocin is greater at temperature optimal for the growth of the producer strain. Growth at elevated temperatures may completely suppress bacteriocin production (53, 173, 270) and sometimes leads to an irreversible loss of the property (53, 150). The latter is most often attributable to loss of the bacteriocinogenic factor (see below).

Maximal bacteriocin yields in a culture may occur at different phases of the growth cycle. Schlegel and Slade (249) showed that streptocin STH_1 production was best during the exponential growth phase, with a sharp decline in the level of bacteriocin before the culture entered stationary phase. By contrast, streptococcin A-FF22 production starts late in the logarithmic phase and activity decreases slowly on prolonged incubation (unpublished observations). Similarly, production of staphylococcin C55 commences in the logarithmic phase, reaches a maximum between 24 and 48 h of growth, and then gradually declines (55). Butyricin 7423 is secreted during the late exponential phase; however, perfringocin 11105 only appeared with the onset of stationary phase and its production or release seemed coincidental with partial lysis of the producer cells (43).

Meitert (197) has reported that some strains of *C. diphtheriae* release bacteriocin continuously whereas others produce it in bursts. The latter effect may signify the spontaneous inducibility of the bacteriocins. Lachowicz (173) studied the dynamics of staphylococcin A-1262a production on solid media. Activity was first detected after 8 h, reached a maximum at 18 to 24 h, and subsequently fell to zero. Other studies have also reported substantial losses of bacteriocin activity on prolonged incubation of cultures (55). This effect may be related to the appearance of specific bacteriocin inactivators or to enzymatic digestion (see below) or, alternatively, could be attributed to readsorption of the bacteriocin to the producer cells.

Aeration of cultures has been found to greatly increase the yield of staphylococcal bacteriocins (51, 85, 153). Mechanical denaturation of the bacteriocins may be minimized by addition of antifoaming agents (153).

An interesting effect is the apparent influence of pH on the recovery of some bacteriocins. In an investigation of conditions for optimal colicin K production it was shown that the control of the medium pH was a critical factor (89). In another study it was found that streptococcin A-FF22 production on Todd-Hewitt agar was increased by adjusting the initial pH of the medium to 6.5 (277).

Inducibility of Bacteriocins

Production of some of the bacteriocins of gram-positive bacteria has been shown to be inducible, in a manner analogous to prophage induction (4). Many of these inducible substances have been shown to be structurally related to bacteriophage components and it has been suggested that these should properly be classified as defective bacteriophages rather than as bacteriocins (86, 233).

Inducible bacteriocins include some that have been detected following the induction of strains of *B. megaterium* (141, 190) and *L. monocytogenes* (105, 106, 269, 290). The production of large quantities of megacin after the irradiation of broth cultures of the producer strain was shown to correlate with lysis of the cells (141). Not all bacteriocins are inducible. Although the production of some bacteriocins may be boosted substantially by induction (43, 291, 292), in other cases, the yield appears to be reduced (51, 128, 186).

Treatments with ultraviolet irradiation or mitomycin C are the methods most often used for bacteriocin induction. A variety of antimetabolic agents were found effective in a study of megacin induction (190). One particular agent may be found to be superior for the induction of the particular bacteriocin under investigation. For example, the yield of perfringocin 11105 was enhanced fourfold after treatment of the producer strain with mitomycin C, but no increase was obtained by use of ultraviolet irradiation, cold shock, or hydrogen peroxide (43).

Production of Bacteriocin Inhibitors and Inactivators

Davie and Brock (61) found that a specific teichoic acid, usually contained within the cell envelope of *S. faecalis* subsp. *zymogenes* strain X14, contributed to the immunity of this organism to its own bacteriocin (hemolysin). Only strains resistant to this lytic agent produced the specific inhibitor. Excretion of some of the inhibitor in the latter stages of the growth cycle resulted in the apparent disappearance of bacteriocin from the cultures. Similarly, the bacteriocin activity in culture supernatants containing staphylococcin 1580 was increased greatly on dialysis, suggesting that these preparations may contain a low-molecular-weight inhibitor of the bacteriocin (153). No characterization of the hypothesized inhibitor was reported.

All of the bacteriocins characterized to date contain a protein component that is essential for biological activity. Irreversible inactivation of some bacteriocins may thus result from digestion by proteases if these are also produced by the bacteriocinogenic organism. This effect was first demonstrated in *Serratia marcescens* (75), but it is also believed to play a role in the inactivation of bacteriocins of *Clostridium botulinum* (69, 157) and group A streptococci (271). Boiling preparations of some heat-stable bacteriocins inactivated the proteases, thus protecting the bacteriocins (69, 271).

LOCATION AND PURIFICATION OF BACTERIOCINS

Because the production of many of the bacteriocins of gram-positive bacteria seems not to be inducible and, for the most part, relatively low titers of activity are detected in cultures, it is necessary for purification purposes to start with large batches of cultures. Some bacteriocins require solid medium for production. Their recovery in solution is generally effected by freeze-thaw elution from the agar (99, 100, 173, 277).

Many of the bacteriocins of gram-positive species seem to exist in both a cell-associated and an extracellular form. The proportion of each form seems dependent on the physiological state of the medium (see below). Bacteriocins in this category include megacin C-216 (68, 124), lactocin LP27 (300), staphylococcin 1580 (153), butyricin 7423 (43), and also staphylococcin C55 and streptococcin A-FF22 (unpublished observations). Similar observations have also been reported for several of the colicins (59).

In the case of bacteriocins that are predominantly cell bound, release from the cell mass can be effected by physical, chemical, or enzymic means. Viridins A, B, and C (bacteriocins of viridans streptococci) may be obtained in a cell-free state after homogenization of bacteriocinogenic cells, but not after sonic treatment or after the use of various chemicals (54). Also, staphylococcin 414 was found to be firmly bound to the cell surface of the producing strain but could be released by mechanical disruption of the cells (84). By contrast, staphylococcin 462 (101) could not be solubilized by fragmenting the producer cells. Most efficient elution was obtained with 7 M urea. Other studies have indicated that staphylococcin 1580 may be extracted from bacterial pellets with 5% sodium chloride (153). Similarly, purification of colicinis E_2-W3110 and E_3-W3110 (117) and bacteriocin JF246 of *S. marcescens* (74) was aided by the extraction of the cell-bound bacteriocin with 1 M sodium chloride. The cell-associated form of streptococcin A-FF22 can be extracted by boiling in dilute acid, the method most often used to obtain preparations of M proteins, the type-specific surface antigens of group A streptococci (76). The streptococcal antibiotics, nisin (311) and diplococcin (216), may also be obtained by acid extraction of the producer cells and may be quite similar substances to streptococcin A-FF22. The bacteriocin produced by strains of phage type 71 *S. aureus* also appears to be extractable by acid (13). In another study (300) it was observed that heat treatment of cultures of a bacteriocinogenic *Lactobacillus* enhanced the release of lactocin LP27 from the cells. An acid extraction effect seems also to be operating in this instance. By using a somewhat different approach Donoghue (68) found that megacins C-216 and C_x-337 were released from megacinogenic cells by lysozyme treatment, combined with trypsin pretreatment. The author concluded that the megacins were probably located on the outer-wall surface.

Studies such as those outlined above point to the possibility that some of the bacteriocins may be cell surface components that are produced in excess under certain conditions and are released from the cells.

After obtaining the crude bacteriocin in a soluble, cell-free form, purification is concerned with the separation of the active entity from other cell components or products and from medium constituents. When working with nondialyzable bacteriocins the use of diffusates of the nutrient medium has the advantage of eliminating all high-molecular-weight, nonbacterial substances from the culture supernatant and thus simplifies purification of the bacteriocin (55, 65, 153).

The methods of bacteriocin purification are those of protein biochemistry and a wide variety of different combinations of procedures have been utilized, with varying success. This is not the appropriate place for discussion of these methods. Because bacteriocins are such an extremely heterogeneous group of substances, specific purification protocols generally need to be empirically designed for each bacteriocin.

Often crude bacteriocin preparations are first concentrated by fractional precipitation with acids, salts, ethanol, or various solvent mixtures. Subsequent purification may be on the basis of size differences (gel chromatography, ultrafiltration, centrifugation) or charge differences (ion-exchange chromatography, electrophoresis, isoelectric focusing).

A common problem is the loss (often massive) of activity as purification progresses. It is im-

portant to monitor the specific bacteriocin activity (units of bacteriocin/milligram of protein) at each step of purification and to modify where possible those steps leading to excessive loss of activity. Protein is generally determined by use of the Folin phenol reagent; however, measurement of differential adsorption at 280 and 260 nm is a convenient method of protein estimation when dealing with multiple samples, as in column fractionation.

ASSAYS OF INHIBITORY AND BACTERICIDAL ACTIVITY

Bacteriocin assays are of many types. In the absence of a chemical test that correlates with the biological activity of the bacteriocin, all assays are based upon the demonstration of antagonistic activity. Not all of them necessarily reflect specific bactericidal action. The titer of a bacteriocin preparation is generally based upon the reciprocal of the highest dilution to cause a particular degree of inhibition of an indicator organism under carefully standardized conditions. Arbitrary units of bacteriocin activity are then often defined as the amount of bacteriocin in 1 ml of the preparation. It is important to exclude nonspecific inhibitory effects. Also, viable producer organisms need to be eliminated from the test preparations by methods such as heat inactivation, filtration, or exposure to chloroform. The method of sterilization should be shown not to alter titer of the bacteriocin.

Heat inactivation of vegetative organisms is generally useful only for crude preparations of extremely heat-tolerant bacteriocins such as streptococcin A-FF22 (271, 277) and staphylococcin C55 (51, 55). Filtration, when used as a sterilizing procedure, may result in losses due to adsorption to the filter (13, 43, 194). Chloroform sterilization must be avoided when assaying certain chloroform-sensitive bacteriocins (35). An alternative method sometimes used for the detection of bacteriocin is the addition of streptomycin to the assay system, coupled with the use of a streptomycin-resistant indicator and a streptomycin-sensitive producer strain (68, 160).

The simplest and most commonly assay used is the critical dilution spot test onto a sensitive indicator lawn. Other assays include those based upon differences in turbidity of indicator broth cultures when grown in the presence of different concentrations of the bacteriocin (116, 153, 248), upon the release of ultraviolet-absorbing material from cells (169), or upon the ability of survivors to bring about the reduction (and color change) of an indicator dye (254).

Inhibition as detected in all of the above assays may be either bactericidal or bacteriostatic. To specifically determine bactericidal units of activity in a preparation it is best to perform viable counts (51, 54, 55, 57, 206).

PROPERTIES OF BACTERIOCINS

A number of physicochemical properties are generally examined to provide information about the composition and structure of bacteriocins (Table 2). Unfortunately, there has been very little standardization of most of the tests used to determine these properties, making direct comparisons between bacteriocins isolated in different studies rather unrealistic. Nevertheless, considerable differences are apparent, even between bacteriocins produced by the same species (or sometimes by the same strain).

Chemical Composition

Bacteriocins are an extremely heterogeneous group of substances. Although chemically diverse, the one unifying property is the presence of an essential protein component.

Tests of sensitivity to specific enzymes (proteinases, lipases, etc.) are often used to help identify important chemical components of bacteriocin molecules. It was on this basis that streptocin STH_1 was thought to be a complex molecule containing essential protein, lipid, and phosphate groups (248).

Chemical analyses indicate that some bacteriocins may be simple proteins (120); however, many others, including certain staphylococcal (84, 101, 149, 153), clostridial (293), and lactobacillus (65, 300) bacteriocins seem, in their present state of purification, to be quite complex molecules with lipid and carbohydrate components in addition to protein. The composition of staphylococcin 414 was likened to that of the staphylococcal cell membrane (84). Investigations of several of the colicins (59) have shown the biologically active protein components to be complexed with lipopolysaccharide antigens on the producer cell surface.

Butyricin 7423 and perfringocin 11105 appear to be amphiphilic proteins (43) and it has been speculated that the hydrophobic regions of the molecules may facilitate interaction with the cell membranes of susceptible organisms. Similarly, Hamon and Peron (111) found that a selection of colicins and enterobacteriocins were all inactivated by anionic detergents. Such bacteriocins were relatively unaffected by cationic detergents and unaffected by nonionic detergents. It was speculated that these bacteriocins may cause local disorganization of the

cytoplasmic membranes of susceptible cells by virtue of their weak detergent activity. Support for this contention was the demonstrated lysis of spheroplasts and protoplasts of sensitive cells by the bacteriocins.

Antigenicity

On the basis of their high molecular weights (generally) and protein composition, it would be anticipated that most bacteriocins would be excellent antigens. However, there are only a few reports in the literature on the antigenicity of bacteriocins produced by gram-positive species. Megacin A-216 has been shown to be antigenic and to evoke an antibody capable of neutralizing its own killing effect (142). Tubylewicz (294) was able to distinguish antigenic differences between four bacteriocins of *C. perfringens* in double-diffusion tests. By contrast, three apparently dissimilar monocins were found to give rise to cross-neutralizing antibodies (106). Studies of different staphylococcins have both failed (84, 101) and succeeded (49) in the demonstration of neutralizing antibodies. Specific immunoglobulin M neutralizing antibodies were produced in rabbits immunized with staphylococcin C55 (49). In addition, nonspecific neutralizing factors for this bacteriocin were found in normal sera from humans, guinea pigs, and some rabbits (49). Failure to produce antibody to streptococcin A-FF22 (271) may possibly be attributed to the low molecular weight of this bacteriocin, but the reason for failures in other cases (84, 101) is less apparent. The antigenicity of bacteriocins produced by gram-positive bacteria warrants further exploration. Studies of antibodies to bacteriocin in human sera may be an interesting area for future investigation.

Physical Properties

Substances that have been designated (perhaps all too casually) as bacteriocins range in size from simple low-molecular-weight proteins, such as streptococcin A-FF22 with a molecular weight of 8,000 (271), to complex defective phage particles with a molecular weight in excess of 10^6. Bradley (25) classified the bacteriocins into two groups, designated as low- and high-molecular-weight bacteriocins. Low-molecular-weight forms are generally more susceptible to trypsin digestion but are less sensitive to heat inactivation. Bradley also suggested that the high-molecular-weight forms were probably phage related whereas the low molecular forms were not. A number of the high-molecular-weight bacteriocins have been examined in the electron microscope and attempts have been made to correlate bacteriocin activity with some visible structure. When strains produce both bacteriocins and phages (or phage components), such studies may be inconclusive or at times even deceptive. A critical issue in bacteriocin definition is the status of particles that are the products of defective prophage (86). Physiologically these substances resemble bacteriocins in that they are unable to propagate within cells susceptible to their killing action. Their production is often inducible on exposure of the host strain to ultraviolet irradiation or mitomycin C.

Gram-positive species, shown to produce bacteriocins that morphologically are identifiable as phage component-like in structure, include *C. botulinum* (133, 178), *Mycobacterium tuberculosis* (132), *L. monocytogenes* (26), and various *Bacillus* spp. (24, 212, 251). Although it has been suggested that all of the defective bacteriophages be excluded from the bacteriocin category, they have been included in the present review for completeness and also because it seems impossible at this stage to distinguish clearly between low-molecular-weight products of defective prophage and true bacteriocins.

Preparations of staphylococcin 414 (84), staphylococcin A-1262a (175), and the so-called killer principle (189), or megacin C_x-337 (68) from *B. megaterium* strain 337, were found to contain ringlike structures of diameter 1.00 to 6.40 pm, which in one study (84) were likened to membrane vesicles.

A not uncommon characteristic of bacteriocins produced by gram-positive species is their apparent existence in two or more distinct physical forms (28, 43, 69, 84, 101, 153, 176, 177, 249, 270, 300). Different molecular species of several of these bacteriocins seem to exist in equilibrium, the relative proportions of the smallest units and of aggregates of these monomers being influenced either by the pH and ionic strength of the preparation (249, 270), or by treatment with 6 M urea or 0.1% sodium dodecyl sulfate (153).

As discussed previously, the distinction between "classical" antibiotics, bacteriolytic enzymes, defective phages, and true bacteriocins is ill defined. Many bacterial products were referred to as bacteriocins before their fundamental nature was determined. Enzymic bacteriocins include megacin A-216, shown to be identical with phospholipase A (215), and the hemolysin (bacteriocin X-14) of *S. faecalis* subsp. *zymogenes* (34). The antibacterial substance produced by *B. cereus* (83) has also been termed a bacteriocin and is believed to be iden-

TABLE 2. *Physicochemical properties of some bacteriocins from gram-positive bacteria*

Bacteriocin	Heat tolerance[a]	Mol wt	Effect of pH	Susceptibility to enzymes		Reference
				Sensitive	Resistant	
Megacin A-216	R 60°C for 30 min S 80°C for 30 min	5.1×10^4	Stable pH 2–7 Unstable pH >7.5	Chymotrypsin Pepsin Trypsin		138, 142
Megacin B-B1	S 60°C for 60 min	5.0×10^4			Trypsin Pepsin α-Chymotrypsin Papain	68
Megacin C-C4MA$^-$	S 60°C for 60 min	1.5×10^5		Pepsin	Trypsin α-Chymotrypsin Papain	68
Megacin C_X-337	S 60°C for 60 min	1.6×10^5		Pepsin α-Chymotrypsin Papain	Trypsin	68
Clostocin A	R 100°C for 30 min	Nondialyzable	Stable pH 4-9	Trypsin Chymotrypsin Pronase P	RNase[b] DNase[c]	128
Clostocin B	S 80°C for 10 min	Nondialyzable	Stable pH 4–9	Trypsin Chymotrypsin Pronase P	RNase DNase	128
Clostocin C	S 80°C for 10 min	Nondialyzable	Stable pH 4–9	Trypsin Chymotrypsin Pronase P	RNase DNase	128
Clostocin D	R 100°C for 30 min	Nondialyzable	Stable pH 4–9	Trypsin Chymotrypsin Pronase P	RNase DNase	128
Boticin E-S5 (small form)	R 100°C for 10 min	$<3.0 \times 10^4$	Stable pH 1.1–9.5 Unstable pH >12.4	Trypsin Chymotrypsin Pepsin	DNase	69
Boticin E-S5 (large form)	R 100°C for 10 min	$>4.0 \times 10^7$		Trypsin Chymotrypsin	Pepsin DNase	69
Boticin P	S 60°C for 30 min	$>4.0 \times 10^6$	Stable pH 6.5–7.5	Trypsin	DNase RNase Alkaline phosphatase Phospholipases C,D	178
Butyricin 7423	R 100°C for 10 min	3.25×10^4	Stable pH 2–12	Trypsin		43
Perfringocin 11105	R 100°C for 30 min	7.6×10^4	Stable pH 2–12	Trypsin		43

Perfringocin a-BP_6K	S 50°C for 15 min	Nondialyzable	Stable pH 4–10 Inactive pH 1–3	Trypsin Papain		292
Perfringocin b-1127	S 55°C for 15 min	Nondialyzable	Stable pH 4–10 Inactive pH 1–3			292
Perfringocin c-541	S 55°C for 30 min		Stable pH 4–10 Inactive pH 1–3			292
Perfringocin d-496	S 55°C for 15 min		Stable pH 4–10 Inactive pH 1–3			292
Bacteriocin 28 of *Clostridium perfringens*	S 55°C for 30 min			Trypsin		186
Lactocin LP27	R 100°C for 60 min	1.24×10^4 [d]		Trypsin Pronase	Ficin	300
Bacteriocin 466 of *Lactobacillus fermenti*	R 96°C for 30 min	Nondialyzable		Trypsin Pepsin	Lysozyme	65
Listeriocins D,S	R 45°C for 30 min S 50°C for 15 min		Stable pH 5–10 Inactive pH 1–4		Trypsin	290
Listeriocins L,W	R 56°C for 30 min S 60°C for 15 min		Stable pH 4.2–10 Inactive pH 1–4		Trypsin	290
Staphylococcin C55	R 100°C for 15 min	$>1.0 \times 10^5$	Stable pH 4–8.5	Pronase Trypsin		55
Staphylococcin 414	R 70°C for prolonged time	1.25×10^{4d}	Stable pH 8	Trypsin Pronase	Ficin	84
Staphylococcin 462	R 50°C for 24 h S 70°C for 3 h	9.0×10^{3d}		Pronase Ficin Chymotrypsin	Lipase	101
Staphylococcin 1580	R 120°C for 15 min	2.0×10^{4d}	Stable pH 3.5–8.5	Trypsin Pronase Chymotrypsin	Lysozyme Lysostaphin	149, 153
Staphylococcin A-1262a	R 100°C for 60 min		Stable pH 1–10		Trypsin Pepsin	173
Streptococcin A-FF22	R 100°C for 60 min	8.0×10^3	Stable pH 2–7 Inactive in alkali	Trypsin Pronase		271
Streptocin STH_1	S 60°C for 10 min	3.0×10^{4d}	Biphasic stability at pH 5 and 10	Trypsin Phospholipase C Alkaline phosphotase	DNase RNase Phospholipase D	248, 249
Streptococcin B-73	S 80°C for 20 min	1.0×10^4		Pepsin Trypsin Lipase	Chymotrypsin Papain α-Amalase	166
Streptococcin B-74628	R 100°C for 60 min	1.0×10^{4d}	Stable pH 2–6.5 Inactive in alkali	Pronase Trypsin		270

TABLE 2—*Continued*

Bacteriocin	Heat tolerance[a]	Mol wt	Effect of pH	Susceptibility to enzymes		Reference
				Sensitive	Resistant	
Viridin B	S 65°C for 30 min	Nondialyzable	Stable pH 5–8	Trypsin Protease	Papain	54
Enterococcin E-1	S 80°C for 20 min	Dialyzable			Trypsin	21

[a] R, Resistant; S, sensitive at indicated temperatures.
[b] RNase, Ribonuclease.
[c] DNase, Deoxyribonuclease.
[d] These bacteriocins exist in more than one form, with different molecular weights; the ones shown are for smallest active form.

tical to the phospholipase A of *B. megaterium*. By contrast, various other bacteriolytic enzymes that are produced by group H streptococci (230, 231), and by *S. aureus* (306) have not been termed bacteriocins. The smallest of the substances to be called bacteriocins include streptococcins A-FF22 (271) and B73 (166), enterocin El A (167), and monomeric forms of streptococcin B-74628 (270), staphyloccins 462 (101), 414 (84), and A-1262a (176), and lactocin LP27 (300), all having molecular weights in the range of 8,000 to 12,500.

Stability

The stability of bacteriocin preparations has often been shown to decrease dramatically with increased purification (69, 185, 270, 271). Addition of bovine serum albumin has been shown to protect some purified bacteriocins from excessive inactivation (153,200). Bacteriocins such as staphylococcin 1580 (149) and also an "antibiotic" produced by *S. aureus* (85) have been found extremely sensitive to mechanical denaturation. Table 2 indicates that bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH. Many of the bacteriocins and bacteriocin-like substances seem considerably more tolerant of acid than alkaline pH extremes (13, 69, 85, 100). Criteria of thermostability of bacteriocins are difficult to define, particularly since this will be dependent upon the state of purification and also upon other factors such as pH, ionic strength, and presence of protective molecules.

GENETIC DETERMINANTS OF BACTERIOCINS

In general, the property of bacteriocin production seems to be an hereditary characteristic of the cell determined by cytoplasmic genes (bacteriocinogenic factors). The genetic determinants of colicins (colicinogenic factors) have been studied in greatest detail. They were one of the three types of episomal elements originally described by Jacob and Wollman (146). More recent studies indicate that most of the bacteriocinogenic factors seem to exist only in the autonomous state within the cell and hence should more appropriately be called plasmids. In the examples investigated it seems that most of the genetic determinants of bacteriocins are plasmid borne. Pneumocin determinants may be an exception. In one apparently unconfirmed study they were shown to be chromosomally located (199).

Bacteriocinogenic factors may determine not only the chemical composition of the bacteriocin, but also the regulation of its biosynthesis, its release from the cell, and the host cell im-

munity to its own bacteriocin. In addition, certain bacteriocinogenic factors have been shown to be associated with fertility genes, capable of promoting the infectious transfer of the plasmid to other strains by conjugation (see below).

One notable characteristic of plasmids is their apparent non-essentiality to the host organism under most circumstances. Spontaneous loss of plasmids may occur periodically in cultures and the observation of instability in some phenotypic trait is often the first indication that a particular gene may be plasmid borne. The irreversible spontaneous loss of bacteriocinogenicity during serial subcultures or long-term storage has been reported for strains of *L. helveticus* (301), *B. megaterium* (124), *S. aureus* (12, 150, 173, 202, 218), group A streptococci (172), and group B streptococci (299). Demonstration of the concomitant loss of one or more other phenotypic characters and also of the irreversibility of the change can be taken as additional indications of plasmid involvement and against alternative interpretations such as point mutations or phase variations.

Often the rate of plasmid elimination is accelerated by exposing the host strain to curing agents or by growing the strain at elevated temperatures. Thus, bacteriocinogenic strains of *S. aureus* (53, 84, 150, 308) have been cured by treatment with acriflavine, acridine orange, rifampin, ethidium bromide, and sodium dodecyl sulfate. Curing of *C. perfringens* type A (134) has been accomplished also after acriflavine treatment. Growth at elevated temperature proved effective for the curing of the bacteriocinogenic strains of *B. megaterium* (68) and *S. aureus* (53, 150, 308). When using curing agents it is vital to include suitable controls to demonstrate if the treatment is merely selecting for proliferation of cells from which the marker has been lost spontaneously. Novick (209) has discussed several criteria that may be useful to indicate the plasmid location of a genetic marker.

Curing of bacteriocin production in staphylococci has been noted to result in altering the resistance of producer cells to the action of the staphylococcin (53). Whereas producer cells were unable to adsorb the bacteriocin and were naturally resistant or immune to its lethal effect, cured strains adsorbed the staphylococcin and were rapidly killed by it. Whether the presence of the staphylococcin plasmid renders bacteriocinogenic cells resistant, immune, or both has not been elucidated (see below).

Transfer of Bacteriocinogenic Factors

Certain bacteriocinogenic factors seem capable of promoting their own transfer on conjugation of the bacteriocinogenic organisms with compatible recipient strains. Infectious transfer of this nature has been demonstrated with some of the colicins (233), but more recently it has also been documented for bacteriocins of streptococci (144, 287) and *C. perfringens* (245). In the latter study intergeneric transfer was indicated, since a strain of *B. anthracis* was the recipient.

Transduction offers an alternative method of plasmid transfer. Analysis of the effect of ultraviolet irradiation of the phage on the frequency of transduction (the Arber effect [10]) may provide useful supportive evidence of the plasmid nature of bacteriocin determinants. Transduction of bacteriocinogenic factors was suggested in two different staphylococcal systems by Vianu (304). The role of transduction in the transfer of genes in vivo is uncertain.

Mindich (199) used the method of transformation to show the transfer of the chromosomally located pneumocin genes between strains of *S. pneumoniae*. By this method he was able to introduce genes determining several different bacteriocins into a single strain. In a recent study (60) high-frequency transfer of bacteriocinogenicity in staphylococci was demonstrated and was thought to be effected by a process of transformation involving plasmid deoxyribonucleic acid.

Immunity to Bacteriocins

A necessary property to ensure the survival of bacteriocinogenic organisms is the presence of specific immunity to the homologous bacteriocin. Bacteriocin immunity is quite distinct from bacteriocin resistance. The latter is determined by the loss of the specific receptor for a particular bacteriocin. Resistant strains are characterized experimentally by their inability to adsorb (specifically) the bacteriocin from solution. Producer cell immunity may be incomplete. Bacteriocin may adsorb to the immune cell and in high concentrations be lethal for its homologous producer strain (181). Immunity breakdown is probably widespread and has been reported for bacteriocins produced by *Corynebacteria* (88), *Mycobacteria* (279), *Clostridia* (246, 291), staphylococci (84), streptococci (87, 166, 271, 297), and *Bacillus* spp. (62, 91, 138).

The nature of producer cell immunity is, in general, poorly understood, but where studied it appears to operate following the adsorption of the bacteriocin to the surface and is probably a consequence of the synthesis of a specific immunity substance. Immunity to megacin A-216 was shown to be retained by protoplasts of bacteriocinogenic strains of *B. megaterium* (214). A highly specific inhibitor of the phospholipiase

A activity of the bacteriocin was isolated from megacinogenic strains (210). This inhibitor was shown to be a protein that acted by slowing down the turnover rate of the enzyme (211). Similarly, the immunity of bacteriocinogenic strains of *S. faecalis* subsp. *zymogenes* to their own bacteriocin (hemolysin) has been attributed to a specific teichoic acid that is found only in strains resistant to the lysin (61). Recently an immunity protein was extracted and purified from cells producing colicin E_3-W3110 and was shown to be capable of blocking the in vitro action of the bacteriocin (147, 257). Formation of a firm complex between a bacteriocin and its specific immunity protein seems to be the mechanism by which bacteriocinogenic cells protect themselves from the lethal action of their own products.

Physicochemical Studies of Bacteriocinogenic Factors

There have been relatively few reports of the isolation and characterization of bacteriocinogenic factors of gram-positive bacteria. Carlton and Helinski (39) were unable to detect any qualitative differences in the deoxyribonucleic acid composition of megacin-producing and nonproducing strains of *B. megaterium*. More recent studies indicate, however, that bacteriocin loss in a strain of *C. perfringens* type A did appear to be associated with the loss of plasmid deoxyribonucleic acid (134). Also, a transmissible plasmid determining the production of the bacteriocin (hemolysin) of *S. faecalis* subsp. *zymogenes* has been isolated and characterized (144).

SPECTRUM OF ACTIVITY

The spectrum of activity of a particular bacteriocin seems to be determined in part by the presence of appropriate, responsive receptors of the susceptible organisms.

Whereas most bacteriocins produced by gram-negative bacteria act on very closely related species, most bacteriocins of gram-positive bacteria exhibit activity against a wide range of gram-positive species (Table 3). Moreover, the effect of some bacteriocins of gram-positive bacteria is not restricted to gram-positive genera; several gram-negative bacteria have been reported to be inhibited by bacteriocins or bacteriocin-like substances produced by gram-positive bacteria. Gram-positive bacteria that have been shown to inhibit gram-negative bacteria include *Lactobacillus acidophilus* (305), *B. cereus* (154), streptococci (54, 312),

TABLE 3. *Activity spectra of some bacteriocins from gram-positive bacteria*

Bacteriocin	No. of strains inhibited/no. of strains tested for various genera								Reference
	Streptococcus	*Staphylococcus*	*Clostridium*	*Corynebacterium*	*Lactobacillus*	*Listeria*	*Bacillus*	*Micrococcus*	
Megacin A-216	0/30	0/10		0/1			51/108	13/14	139
Clostocin A	0/1	0/1	4/14				0/22	0/1	128
Clostocin B	0/1	0/1	14/14				22/22	0/1	128
Clostocin C	0/1	0/1	14/14				22/22	0/1	128
Clostocin D	0/1	0/1	10/14				0/22	0/1	128
Welchicin D366	7/141	0/112	114/117	10/73			146/221		246
Welchicin 58/65	5/141	0/112	114/117	0/73			0/221		246
Welchicin P24	0/141	0/112	107/117	0/73			0/221		246
Lactocin LP27	0/2	0/4			3/5		0/3		300
Mutacin MT118	50/50	10/10			5/5		4/4	2/2	104
Mutacin BHT	32/50	4/10			3/5		1/4	2/2	104
Enterococcin E-1	154/372	0/137					0/3		21
Enterococcin type 1	16/19	2/2	1/1	2/2	2/2		4/5	2/2	35
Enterococcin type 2	11/18	0/2	0/1	1/2	2/2		0/5	0/2	35
Enterococcin type 3	12/18	0/2	0/1	1/2	0/2		0/5	1/2	35
Enterococcin type 4	9/17	0/2	0/1	0/2	0/2		0/5	1/2	35
Enterococcin type 5	5/15	0/2	0/1	0/2	0/2		0/5	0/2	35
Listeriocins D,S	0/3	0/4		0/9		3/8			290
Listeriocins L,W	0/3	0/4		0/9		1/8			290
Staphylococcin 414	2/3	54%[a]			1/1		2/3	2/3	84
Staphylococcin 263	1/3	25%[a]			0/1		1/3	0/3	84
Staphylococcin 462	1/3	6%[a]			0/1		1/3	1/3	84
Staphylococcin 462	5/8	3/19		1/1	2/8		8/15	1/5	102
Staphylococcin C55	4/4	20/29		4/5		1/1	4/4	1/1	51
Staphylococcin 1580	19/22	30/91	0/1	2/2	0/1	1/1	6/9		149
Streptococcin A-FF22	9/22	1/5		2/3		0/1	3/4	2/2	277
Streptococcin B-74628	145/278	4/29		0/2		0/1	3/5	4/4	270
Streptococcin B-73	34/49	2/20		2/10		1/10			166

[a] Strains inhibited/strains tested not given.

staphylococci (129, 221), and corynebacteria (197).

Furthermore, stable L-forms (but not the parental bacterial forms) of some gram-negative bacteria were found to be sensitive to the bacteriocin (hemolysin) of *S. faecalis* subsp. *zymogenes* (113, 156), the implication being that the cell wall had exerted a protective effect against the bacteriocin. The broadness of the activity spectra, including in some instances species of a different Gram reaction, is another interesting characteristic distinguishing bacteriocins of gram-positive from those of gram-negative bacteria.

Studies of the activity spectra of different bacteriocins against strains of the same and of heterologous species have aided differentiation between bacteriocins and have led to the establishment of bacteriocin typing procedures for use in epidemiological studies (see below).

MODE OF ACTION OF BACTERIOCINS

Most of the original information regarding the mechanism of action of bacteriocins has been based on studies of several of the colicins. Nomura and his group have been prominent in this field and much of the early literature was reviewed by Nomura in 1967 (207). Research on this subject has largely focused upon two distinct aspects of bacteriocin action on susceptible bacteria: the kinetics of the physical interaction between bacteriocin and susceptible cells, and the detection of specific biochemical lesions within the affected organisms. In a widely accepted hypothesis of the mode of action of bacteriocins, it was suggested that the interaction of a bacteriocin with a sensitive cell occurs in two stages (223). The first stage corresponds to the physical adsorption of bacteriocin molecules to exposed cell-envelope receptors and is probably a reversible phase. No permanent physiological damage is produced and removal of the bacteriocin during this stage apparently leaves the cell unscathed. At a measurable time later the second stage develops in which irreversible pathological changes are effected via specific biochemical lesions.

Kinetic data indicate that bacteriocins behave as particulate lethal agents, killing sensitive bacteria in what amounts to a single-hit process. This so-called "quantal" killing (184) may be interpreted to indicate that a single bacteriocin particle can, with a certain probability, kill a sensitive cell. Quantal killing was distinguished from the "molar" (cooperative) killing action of the "classical" antibiotics. Studies of the colicins showed that they adsorbed to specific receptors on the external envelope of the organism and that their lethal action appeared to be directed from this external site. Evidence for this concept was based largely on the demonstrated rescue of cells that had adsorbed a lethal dose of bacteriocin by removal of the attached bacteriocin with trypsin treatment (223).

Luria (183) and Nomura (206) independently proposed similar models to account for the dramatic lethal action effected by a single molecule attached to the surface of a bacterial cell. It was suggested that attachment of a lethal unit of bacteriocin to its specific receptor causes a reversible change which is then transmitted and amplified via the cell envelope to membrane-bound biochemical targets. Changeux and Thiery (41) postulated that if the bacterial cell membrane be interpreted as consisting of a complex arrangement of repeating protomer units, then conformational changes in these units, induced by attachment of the bacteriocin, may be sequentially propagated throughout the membrane. Some supporting evidence for the bacteriocin-induced modification of the structure or conformation of the cell envelope has been obtained by use of fluorescent probes such as *N*-phenyl-1-naphthylamine. Increases in the fluorescence of the bound probes after treatment of sensitive cells with colicin E1 (45), colicin A-C31 (151), or staphylococcin 1580 (151) were interpreted to reflect bacteriocin-induced structural changes in the cell envelope.

The recent findings that colicin E3 (20, 23, 198) and perhaps also colicins E2 (234) and K (278) may interact directly with their specific biochemical targets in cell-free systems have cast doubt on the universal applicability of the above model (258). The lethal action of many of the bacteriocins may, it seems, be attributable to direct enzymatic activity or to the activation of endogenous suicidal enzymes. Transition between the two previously described stages of bacteriocin action may represent the time required for penetration of the bacteriocin into the cell envelope. Once having penetrated the outer membrane, trypsin (223) or serum (191) rescue of the cells may then be impossible. The following discussion of various aspects of the mode of action of bacteriocins shall be largely confined to data relating to gram-positive organisms, comparisons being made with other bacteriocins where appropriate.

Adsorption of Bacteriocins

The adsorption of bacteriocins is generally demonstrated indirectly by the drop of bacteriocin titer in solutions after mixing with an excess of susceptible bacterial cells. The impor-

tance of using a sufficiently low concentration of bacteriocin in such tests to clearly detect adsorption was stressed in studies utilizing lactocin LP27 (301), megacin A-216 (121), and staphylococcin C55 (57).

To help establish that the removal of bacteriocin activity from solution is due to adsorption and not to enzymic inactivation it may sometimes be possible to recover the active bacteriocin from the treated cells by elution processes (Tagg, unpublished observations). Alternatively, use has been made of heat-killed cells (or cell walls), apparently free of enzyme activity, to demonstrate adsorption (57).

Although in many cases the adsorption of bacteriocins has been shown to be highly specific for susceptible bacteria (8, 57), other bacteriocins, such as staphylococcins 414 (84) and 1580 (149), lactocin LP27 (300, 301), and streptococcin B-74628 (270) appear to lack this adsorption specificity. Each of these bacteriocins has been shown to adsorb to bacteria that are resistant to its killing action. This nonlethal binding may be a reflection of the high surface activity of some bacteriocins that makes them capable of adsorbing nonspecifically to various bacteria, as has been reported for some of the polypeptide antibiotics such as polymyxin B (192).

Although adsorption of bacteriocins has been shown to exist in most instances, and may be specific or nonspecific, in a few instances no adsorption to susceptible or to resistant bacteria could be demonstrated. Examples of inability to show adsorption of bacteriocins were reported for staphylococcin 462 (102), for bacteriocin 28 of *C. perfringens* (186), and for viridin B (54). At least in the case of viridin B, failure to demonstrate adsorption was shown not to be due to the use of insufficient low levels of the bacteriocin.

Development in the investigation of the cellular location and chemical nature of bacteriocin receptors is still somewhat embryonic, especially relating to gram-positive bacteria. The precise location of colicin receptors has been the subject of considerable controversey, with some evidence pointing to a cell wall location (240, 309) and other studies strongly implicating the cytoplasmic membrane (18, 261, 262). In view of the great diversity in the composition and structure of bacteriocin molecules it seems plausible that receptors for different bacteriocins may be of various compositions and may occur in different locations.

Colicins have been shown to be active against stable L-forms of susceptible bacteria (262) and also against isolated membrane vesicles (18) and disrupted spheroplasts (278). Similarly, protoplasts or L-forms have been shown to be susceptible to boticin E-S5 (8, 69), megacin A-216 (140), and several staphylococcins (148–150). Isolated membrane vesicles were also shown susceptible to staphylococcin 1580 (151). Such studies indicate the effectiveness of bacteriocins against organisms lacking a cell wall and emphasize the possible importance of direct contact with the membrane to effect bacteriocin action in these cases.

It has been suggested by Sabet and Schnaitman (241) that certain regions of the cell membrane that were shown by Bayer (15) to exist in intimate contact with the cell wall may offer potential sites of bacteriocin attachment. Naturally resistant bacteria or resistant mutants may have access to their membrane receptor regions physically blocked by outer components of the cell envelope (18, 260). Support for this contention has been derived from the observations of inhibition of L-forms derived from bacteriocin-resistant strains by staphylococcin 1580 (151), colicin A-C31 (151), and a bacteriocin of *S. faecalis* (156). This is a highly controversial area of study and for the present the question of the location of bacteriocin receptors remains unsettled.

Recently it has been reported that proteinaceous receptors for colicin E3-CA38 (241) and colicin M-K12 (31–33) have been identified and purified. In the latter case the protein was found to be the receptor for phage T5 also, and it was isolated as a single polypeptide chain of molecular weight 85,000 (33). In addition, lipopolysaccharide components of the cell envelope are thought to be associated with receptor activity for colicins B (94) and E_2-W3110-52 (40). The colicin E receptor may function also as a receptor for vitamin B_{12} (67) and for phage BF23 (38). Similarly, the receptor for colicin K-235 may function also for the adsorption of polymyxin B (192). Thus it seems that certain of the bacteriocin receptors may be multifunctional, offering (in the case of coadsorption of vitamin B_{12}) a possible rationale for the evolutionary retention of a substance facilitating interaction of the cell with a lethal agent. Further investigations of the bacteriocin receptors may prove particularly rewarding, especially as it relates to the structure and function of biological membranes.

Bacteriocin-Induced Cell Damage

The physiological state of the indicator culture has been shown to have a strong influence on susceptibility to the lethal action of bacteriocins. Actively multiplying cells were most sensitive to streptococcin A-FF22 (271), staphyloc-

cin 1580 (152), bacteriocin 28 of *C. perfringens* (186), and bacteriocin E-1 (22) and bacteriocin X-14 (hemolysin) (14) of *S. faecalis* subsp. *zymogenes*. This indicates a requirement for active cellular metabolism to effect the killing of the cells. A related effect seems to be the observed correlation between the temperature of incubation and the rate of kill by bacteriocins such as streptococcin A-FF22 (271), megacin A-216 (121, 140), and staphylococcin C55 (52). Jetten and Vogels (152) reported that cells pregrown at 37°C were more sensitive to staphylococcin 1580 than were cells pregrown at 20°C. Also, the lethal effect of the staphylococcin appeared pH dependent, with optimal killing at pH 7 to 8 (152).

Kinetic data indicate that staphylococcin 1580 (152), streptocin STH_1 (249), megacin A-216 (120), and megacin C-C4MA$^-$ (123) kill sensitive cells by single-hit kinetics, similar to that demonstrated for several of the colicins (233). These results are said to indicate that a single bacteriocin molecule may be sufficient to kill a sensitive cell. However, it has been estimated that a 50% kill of indicator cells can occur at a concentration of 136 molecules of megacin A-216 (120) or approximately 440 molecules of staphylococcin 1580 (152) per treated cell. This has been interpreted to indicate that there is a relatively low probability that an adsorbed bacteriocin molecule will exert its lethal effect, possibly due to the presence of a number of nonlethal binding sites in the cell envelope (152).

The apparent rescue of bacteria preexposed to potentially lethal doses of bacteriocin by treatment with trypsin has been taken as supporting evidence of a two-step killing action of bacteriocins, and has often been interpreted as showing that bacteriocins exert their lethal effect from their surface attachment site (223). Trypsin-sensitive bacteriocins that have been shown to be susceptible to trypsin rescue effects during the early stages of their interaction with sensitive bacteria include staphylococcin C55 (57) and enterocin-E1A (167). In the latter example, rescue could only be demonstrated in the first 2 to 3 min after exposure to the bacteriocin indicating to the authors that the bacteriocin may either be rapidly taken up by the cells or that it may no longer be sensitive to trypsin when cell bound. Failures in other studies to show trypsin rescue of cells treated with boticin E-S5 (69), staphylococcin 1580 (152), and bacteriocin 28 of *C. perfringens* (187) may be attributable to similar effects. It seems clear that trypsin is only able to restore colony-forming ability to bacteriocin-treated cells when added before the development of functional damage (223). Donoghue (68) found that even though megacins C-216 A$^-$ and C_x-337 are both resistant to trypsin, partial rescue could be effected by trypsin treatment of organisms that had adsorbed a lethal dose of megacin. The interpretation offered was that the megacin had been removed due to the digestion of its adsorption site on the surface of the cells.

Specific biochemical targets of a number of the bacteriocins produced by gram-positive bacteria have now been identified (Table 4). Principal lesions occur generally in energy production, macromolecule synthesis, or membrane transport and permeability. These may then trigger various secondary metabolic disorders. Most of the effects are similar to those associated with different colicins. Some more unusual phenomena that have been attributed to bacteriocins of gram-positive bacteria are bacteriolysis, bacteriostasis, sporostasis, and spheroplast formation.

As described previously, both megacin A-216 and the enterococcal bacteriocin, X-14, have been shown to be enzymes having bacteriolytic activity. Other bacteriocins shown to have bacteriolytic action (but not characterized as enzymes) include some of the monocins (109), perfringocin 11105 (43), and boticin E-S5 (8, 157). In the case of boticin E-S5, the lytic effect was subsequently said to be secondary to the primary cell lesions (70). Viable cells were necessary for the demonstration of lysis by megacin A-216 (140) and boticin E-S5 (8). The only colicin to have primary bacteriolytic activity appears to be colicin M (31).

The inclusion within the bacteriocins of substances having only a bacteriostatic effect on other bacteria may be inappropriate. Bacteriocins said to have bacteriostatic activity include boticin P-PM15 (178), bacteriocin 28 of *C. perfringens* (187), lactocin LP27 (301), and staphylococcin 462 (102). Another bacteriocin, viridin B (54), has been shown to exert a bactericidal effect against a *Neisseria sicca* strain, with only a bacteriostatic effect against a coagulase-negative staphylococcus. Labeling a bacteriocin as bacteriocidal or bacteriostatic may be at times premature until a wide range of indicator strains is tested. It is also possible that bacteriocin preparations exerting such different effects may represent two or more substances with varying capacities to inhibit or kill different indicator strains.

Bacteriocins with sporostatic action include boticin P-PM16 (178), boticin E-S5 (8), and the bacteriocin produced by *C. perfringens* strain BP6K-N_5 (252). Also in this category is the

TABLE 4. *Biochemical effects of bacteriocins from gram-positive bacteria on sensitive cells*[a]

Bacteriocin	Inhibition of synthesis of			Degradation of		Other effects	Reference
	DNA	RNA	Protein	DNA	RNA		
Megacin A-216	NT	NT	NT	NT	NT	Leakage of UV-absorbing material, lysis of protoplasts	140, 215
Megacin C-C4MA$^-$	+	+	+	+	−	Induction of lysogenic phage	122, 123
Bacteriocin 28 of *Clostridium perfringens*	−	−	−	−	−	Conversion to spheroplasts	187
Lactocin LP27	−	−	+	−	−	Inhibition of active K^+ transport	301
Staphylococcin C55	+	+	+	−	+	No cell lysis	52
Staphylococcin 462	+	+	+	−	−	Inhibition of ATP production	102
Staphylococcin 1580	+	+	+	−	+	Leakage of UV-absorbing material; inhibition of: ATP production; active transport of ions and amino acids; glucose incorporation into acid-precipitable and glycogen-like material	148, 151, 152
Streptococcin A-FF22	+	+	+	−	+	Inhibition of glucose incorporation into acid-precipitable and glycogen-like material	271
Streptococcin STH_1	+	+	+	−	−	Inhibition of active Rb^+ transport	250
Enterocins E1A and E1B	+	+	+	−	−	E1A inhibits isoleucine accumulation and induces efflux of previously accumulated isoleucine	167

[a] NT, Not tested; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; UV, ultraviolet; ATP, adenosine 5'-triphosphate.

bacteriocin-like "autoantibiotic" of *B. laterosporus* (266).

Bacteriocin 28 of *C. perfringens* acts on the cell wall of viable indicator cultures, converting them to spheroplasts, which under appropriate conditions are then capable of growing as L-form colonies (187). Colicin M has also been shown to induce the formation of spheroplasts in the presence of 16% sucrose (31).

There have been relatively few reports of the nature of the morphological changes produced in susceptible bacteria on treatment with bacteriocins. Clawson and Dajani (44) showed that extensive structural changes occurred in a group A streptococcus strain after exposure to staphylococcin C55. Morphological changes observed included the condensation of nuclear material, partial loss of ribosomes, modification of mesosomes, and eventual dissolution of the cell contents. No effects on the cell wall were noted. Similar changes were observed in sensitive bacteria treated with streptococcin A-FF22 (275) and boticin E-$S5_1$ (70).

APPLICATION OF BACTERIOCINS IN BACTERIAL CLASSIFICATION AND TYPING

Bacteriocins have found widespread application in epidemiological studies as specific marker properties of bacteria. Various typing schemes have been established that are based upon either the production of or sensitivity to a range of different bacteriocins. More precise fingerprinting of strains may be obtained by combination of both methods, sometimes in collaboration with other methods such as phage and serotyping. Because of the relatively narrow spectra of bacteriocins, bacteriocin typing has been most successfully used in studies of gram-negative bacteria but the principles are now finding more extensive application to the typing of gram-positive species.

The bacteriocin-like activity of an epidemic strain of *S. aureus* was used as a marker for the rapid screening of staphylococci from cases and carriers and was said to have aided the man-

agement of the outbreak (9). Pulverer and Sieg (228) were able to type 50% of 300 strains of *S. aureus* on the basis of their production of 53 distinct reaction patterns on 12 indicator strains of *S. aureus*. In another study Ivanov (135) classified 93.3% of 721 strains of staphylococci into six groups according to their susceptibility to a set of seven staphylococcin preparations. Typing on the basis of sensitivity to bacteriocins is often, as indicated in the above example, associated with a higher proportion of typable organisms.

Group D streptococci may be typed according to the production of (297) or the sensitivity to (225) bacteriocins. Combined enterocin production and sensitivity typing (159), using six standard indicator strains and six standard producer strains, respectively, enabled the division of 130 strains of *S. faecalis* into 25 groups. Combined bacteriocin production/sensitivity typing has also been used for the fingerprinting of human oral streptococci (162). By use of 10 producers and 10 indicators it was possible to demonstrate individual patterns of reactivity associated with 38 of 40 strains tested.

Typing of *C. perfringens* according to bacteriocin sensitivity gave 81.33% typable with use of four bacteriocins (244) and 98.9% typable using 10 bacteriocins (185). Twelve (244) and 50 (185) distinct bacteriocin types were recognized in these two studies.

Classification of some rapidly growing mycobacterias has been shown to be possible on the basis of the production of species-specific bacteriocin inhibition patterns (279). The mycobacteriocin activities correlated well with other methods of classifying and differentiating these organisms. Schema of bacteriocin typing on the basis of production of distinguishable patterns of reactivity have also been established for the labeling of *M. tuberculosis* (280), *C. diphtheriae* (88), and viridans streptococci (222). More widespread application of these procedures, with extension to other species, seems assured, particularly where deficiencies exist in the established methods of typing.

BACTERIOCIN ACTIVITY IN VIVO

Teleologically, it is very tempting to assume some major role for bacteriocins and bacteriocin-like substances in the regulation of population dynamics in various bacterial ecosystems. One possible basis for this assumption is the seemingly universal occurrence of bacteriocins in the bacterial world and the recognition that to have so consistently survived the pressures of evolution they must fulfill some vital function. Reeves (232, 233) has discussed some ideas relating to the bacteriocins' "raison d'etre" and suggested that bacteriocin receptors (a structure leading to cell death) must surely have some essential alternative function for the bacterium. He suggested that this function may be a recognition system related to the cross-fertility potential of different strains.

Studies of the factors contributing to the maintenance of alteration of bacterial populations within microecosystems are rare. Although by extrapolation from observations of in vitro interactions it would seem reasonable for bacteriocins to play some major role, this has in fact seldom been clearly demonstrated. Undoubtedly many bacteria are capable of displacing or suppressing the growth of established resident bacteria in the indigenous microbiota. This was recognized very early in the development of bacteriology and indeed was a driving force behind many of the early investigations in this field. It seemed, on the basis of clinical and epidemiological observations, that certain inhibitory organisms of low disease potential were capable of displacing or preventing the establishment of pathogens such as *B. anthracis* and *C. diphtheriae* (72, 73). With the advent of the antibiotic era the application of microorganisms as prophylactic and therapeutic tools was largely discontinued. Interest in this approach has been revived recently since it may be an ecologically more valid means of pathogen control than the use of antibiotics, which have widespread destructive effects on the resident microbiota.

The relatively avirulent strain 502A of *S. aureus* has been used to control outbreaks of staphylococcal infection in nurseries and to treat patients with recurrent staphylococcal furunculosis (7, 256). This strain is capable of interfering with the growth of other stains of *S. aureus* both in vivo and in vitro. The mechanism of this interference phenomenon has not been established. Attempts to demonstrate a bacteriocin that may play a role in this phenomenon have been unsuccessful; however, it remains a possibility that certain bacteriocins may only be effectively produced under the highly specialized conditions existing in vivo. Experimental mixed infections with interfering and susceptible bacteria indicate that active multiplication of the antagonistic organism is essential for the elimination of the target bacterium (6, 243). Perhaps, if bacteriocins are responsible, they are active only for a limited period due to inherent instability or to inactivation by intrinsic factors.

Gerasimov (87) and also Sanders (243) have pointed to the possible role of alpha-hemolytic

streptococci within the normal microbiota of the throat in resistance to infection by group A streptococci. Prospective studies (46) indicated an inverse correlation between the in vitro inhibitory activity of the resident bacteria and subsequent rates of infection with group A streptococci. Although the mechanism of the observed interference was not well defined, the recent observations of Dajani et al. (54) suggest that such interference may well be a function of bacteriocins produced by the viridans streptococci.

What then is the evidence that bacteriocins may have some role in microbial interaction in nature? Only a few studies have succeeded in the demonstration of bacteriocin production during experimental infections of animals. Antibiotic activity was recovered from peritoneal washings of mice that had been infected intra-abdominally with antibiotic-producing streptococci (255). Others (30, 98) have found colicin-like activity in the sera of animals infected with colicinogenic strains and claim that in this manner bacteriocins may contribute to the bactericidal action of blood.

In several studies strains shown to produce well-characterized antibiotics or bacteriocins in vitro were also found to eliminate susceptible bacteria in experimental mixed infections. An antibiotic-producing strain of *S. albus* gave strong protection against *C. septicum* in mice (96) and later it was shown (97) that the partially purified antibiotic was also protective in vivo. Similarly, it was found that a bacteriocinogenic enterococcus was capable of inhibiting, without completely eliminating, a susceptible strain of *S. faecalis* in mixed infections of the rat kidney (95). In another study, guinea pigs were protected against up to 5 lethal doses of *C. perfringens* by the concomitant injection of an inhibitory enterococcus (168).

It has been suggested that bacteriocins of phage type 71 *S. aureus* may have some role in mixed skin infections as evidenced by the elimination or reduction of group A beta-hemolytic streptococci when these sensitive organisms are inoculated in the skin of the hamster together with the bacteriocinogenic staphylococcus (56). In this instance, bacteriocinogenicty would be theoretically advantageous for the staphylococcus invading a skin lesion where the group A streptococcus may already be established. Although this may explain why streptococci and phage type 71 staphylococci are rarely found in the same lesion (50), little epidemiological information is available to support this point aside from the observation that phage type 71 staphylococci are usually found in pure culture in bullous impetiginous lesions (66).

Some experimental evidence indicates that bacteriocins probably do not play a significant role in certain microenvironments, such as in dental plaque. Streptococcal bacteriocins have been shown to be inactivated by proteases found in dental plaque and saliva, and bacteriocinogenic and susceptible streptococci have been recovered simultaneously in the same sample (161). Furthermore, a coating of extracellular polysaccharides, substances comprising a large proportion of the bacterial matrix in dental plaque, renders normally susceptible organisms resistant to the action of *S. mutans* bacteriocin (237).

A theoretical objection to the use of bacteriocins or bacteriocinogenic bacteria as an antibacterial weapon in treatment or prophylaxis is their ability to stimulate neutralizing antibody (49). In one study (298), colicin-neutralizing activity (thought due to antibody) was found to occur in the sera of 12 of 21 elderly bacteriuric and nonbacteriuric subjects. Also, nonspecific neutralizing factors exist in certain sera (49). It would seem that the only bacteriocins likely to have therapeutic application would be those that are not strongly antigenic.

Lachowicz (173) has used staphylococcin A-1262a to treat, with local injections, 50 patients having a variety of staphylococcal lesions. He claimed good results with complete recovery in 42 of the patients.

It was shown in assays of potency that purified colicins V and K had similar inhibitory activity on a per weight basis to that of the therapeutic antibiotics kanamycin, streptomycin, and oxytetracycline (195). Further indications of the relatedness of bacteriocins and antibiotics may be drawn from experiments that showed that colicin K pretreatment of cells made refractory to the colicin may protect these cells against lethal doses of polymyxin B (192). One possible interpretation is that the colicin and polymyxin B may share a common surface receptor.

When contemplating the use of bacteriocins in clinical situations one important consideration is their possible pathological effects. Recent studies indicate that colicin E3 has a direct effect on ribosomes, not only from bacteria, but also from mouse ascites cells (295). Also colicin E2 inhibits the multiplication and chlorophyll synthesis of *Euglena gracilis* (259), and staphylococcin A-1262a has been shown to interfere with the growth of *Trichomonas vaginalis* (173). Studies using partially purified preparations of streptococcin A-FF22 indicated specific toxicity for beating-heart cells in tissue culture

preparations and led to speculation of a possible role for this bacteriocin in the pathogenesis of rheumatic carditis (273). Preliminary experiments with highly purified streptococcin A-FF22 have failed to confirm this specific toxicity for heart tissue (Tagg, unpublished observations).

Montgomery and associates (201) observed a possible association between bacteriocin production by *S. faecalis* subsp. *liquefaciens* and the ability to produce hematogenous pyelonephritis in the rat. They suggested that the bacteriocin may be toxic to kidney tissue or, alternatively, may depress normal defense mechanisms. No additional evidence was reported. Other reports have indicated that there may be a relationship between the carriage of bacteriocinogenic factors and virulence of certain strains of *Pasteurella pestis* (36, 37) and of *Escherichia coli* (264). In the latter study it was remarked that strains of *E. coli* associated with bacteremia in man and animals commonly produced colicin V. In another study Warren and associates (308) demonstrated that genetic information for both bacteriocin and exfoliative toxin production by a phage group II staphylococcus was jointly eliminated after growth in the presence of ethidium bromide or at elevated temperatures. It was considered that although the bacteriocin and toxin seem to be distinct proteins they may be coded by the same plasmid in certain strains. An alternative explanation may well be that the curing agents simultaneously eliminated two plasmids, each controlling a distinct biological activity.

BACTERIOCINS OF VARIOUS GRAM-POSITIVE GENERA

This section is intended to provide an outline showing the development and current state of knowledge regarding the bacteriocin-like substances produced by strains of the various gram-positive genera. Its purpose is to serve as a guide to some of the more significant literature rather than to provide in-depth data from individual studies.

Bacillus

Strains of many of the *Bacillus* spp. have been shown to produce antibiotic substances. Many are poorly characterized but undoubtedly some are bacteriocin-like in nature.

Several species have been reported to produce "antibiotics." These include *B. circulans* (203), *B. polymyxa* (77), *B. laterosporus* (266), *B. pumilus* (2, 82), and *B. cereus* (154). *B. cereus* has also been shown to produce a bacteriolytic principle (208), which may be identical to phospholipase A (83). Other studies of *B. cereus* have categorized the inhibitory substances as bacteriocins (91, 107). Additional studies are indicated to help elucidate the relationship between these different inhibitors. Other *Bacillus* spp. that have been reported to have bacteriocin-like inhibitors are *B. stearothermophilus* (253), *B. licheniformis* (25), *B. thuringiensis* (62, 91, 302), and *B. subtilis* (25, 86, 212). The studies of *B. subtilis* and *B. licheniformis* have indicated that the bacteriocins are of the defective phage type.

The most extensively studied and the best characterized of the bacteriocins of the *Bacillus* spp. are those produced by *B. megaterium*. The first report of the antibacterial principle (megacin) produced by *B. megaterium* strain 216 was by Ivánovics and Alföldi in 1954 (138). A large number of publications dealing with the characterization of megacin soon followed and these have been summarized previously (136, 137). It seemed that megacin had many of the characteristics of a bacteriocin, including production by lethal biosynthesis, a narrow spectrum of activity, and a proteinaceous composition. Observation that megacin was bacteriolytic in its action raised the question (140), however, that it may be an enzyme. Some time later Ozaki and associates established in fact that this megacin was identical to phospholipase A (215). The recognition that there are a number of quite different megacins (124, 189) has led to the formulation of a system of classification that is based on the production characteristics, activity spectra, and mode of action (124). The original megacin, produced by strain 216, became known as megacin A, and megacin types B and C were also identified. Another megacin that has production characteristics similar to megacin C, but a different mode of action, has variously been referred to as killer principle (189) or megacin C_x-337 (68).

Clostridium

Perhaps the first reference to the production of inhibitory substances with bacteriocin-like properties by clostridia occurred in a study of phage production by *C. perfringens* (263). Subsequently, there have been reports of bacteriocin production by strains of *C. botulinum* (16) and related nontoxigenic strains (157), and also by *C. sporogenes* (16, 17), *C. butyricum* (43), and various nonpathogenic clostridia (127, 128). The best characterized, and most extensively studied, are bacteriocins of *C. botulinum* and *C. perfringens*.

Strains of *C. botulinum* belonging to toxin

types A, B, and C were shown to produce a variety of bacteriocins that were unrelated to the type of toxin or in fact to the ability to produce toxin (16). In another study using electron microscopy, toxin type B and E strains were shown to produce bacteriocin-like substances having the structure of phage tails (133). Similarly, it was demonstrated that boticin P-PM15 produced by a nontoxigenic strain was also of the defective phage particle type (178). The bacteriocin was shown to be heat labile and trypsin sensitive. By comparison, boticin E-S5, a bacteriocin shown by Kautter and associates (157) to be produced by a similar nontoxigenic strain, differed from boticin P-PM15 in being dialyzable and stable to heating. Later Ellison and Kautter (69) reported that strain S5 appeared to produce two distinct boticins, the larger of which had a molecular weight greater than 4×10^7. Because the morphological structure of this bacteriocin was not studied, its relationship to the defective phage type of bacteriocins is uncertain. Initial studies demonstrated that both of the bacteriocins produced by strain S5 were bacteriolytic (8, 69); however, it was suggested later that this may be a secondary effect (70).

Săsărman and Antohi (244) initiated studies of the bacteriocins of *C. perfringens* in 1963. They found four strains producing bacteriocins with different activity spectra among 237 strains tested. Tubylewicz (291) reported five different bacteriocins produced by five of 35 type A *C. perfringens* strains. Subsequent studies of four of these bacteriocins provided data on the physiochemical (292), antigenic (294), and chemical (293) characteristics. It is clear that *C. perfringens* strains produce a large number of different bacteriocins that are distinguishable by examination of activity spectra (246). Ten different bacteriocin preparations were used in a provisional typing schema based on patterns of bacteriocin susceptibility of various strains of *C. perfringens* (185).

Mahony and associates have studied one of the bacteriocins of *C. perfringens* in some detail (186). Investigation of its mode of action indicated that it seemed bacteriostatic and that it caused conversion of indicator strains to spheroplasts, either by removing the existing wall or inhibiting cell wall synthesis (187). More recently an independent study (43) reported that perfringocin 11105 may have an effect on an indicator strain similar to that reported above. Perfringocin 11105 and a bacteriocin produced by *C. butyricum* (butyricin 7423) have been highly purified and have been found to have the properties of amphiphilic proteins (43).

Corynebacterium

The production of bacteriocin-like inhibition by strains of various *Corynebacterium* spp. was documented by Thibaut in 1949 (283–285) and subsequently confirmed by Terrasse and Sohier (282). In collaboration with Fredericq, Thibaut was able to demonstrate that, according to differences in activity specta, several different antibiotics appeared to be produced by strains of *C. diphtheriae* type gravis (286). A typing method, developed to supplement phage typing, was based upon determining differences in sensitivity to three antibiotic-producing strains. More recently a typing schema relying on the detection of different bacteriocins produced by the test strains has been developed by Gibson and Colman (88) with promising results. In none of the studies referred to above nor in those reported from Russia (170, 171) or from Romania (196, 198) has isolation and characterization of the bacteriocins been documented. Clearly this is an area demanding further study.

Lactobacillus

Various lactobacilli have been shown to produce antibacterial substances that are quite unrelated to bacteriocins but which may, nevertheless, mimic the action of bacteriocins on cursory examination. These include hydrogen peroxide (310), lactic acid (288), and the broad-spectrum antibiotic lactocidin (305).

Well-characterized bacteriocin-like substances produced by lactobacilli have been studied by De Klerk and associates (63–65) and by Upretti and Hinsdill (300, 301). Representative strains of both homo- and hetero-fermentative species have been found to be bacteriocinogenic (63, 64). Whereas a number of different bacteriocins having distinct activity spectra were produced by homofermentative lactobacilli (64,300), it seemed that only one type of bacteriocin was identifiable within 25 bacteriocinogenic *L. fermenti* cultures (63). Bacteriocins produced by *L. fermenti* strain 466 (65) and by *L. helveticus* strain LP27 (300) have been purified and characterized, and a number of similarities in chemical composition and physicochemical properties have been discussed (300). Both were isolated as macromolecular lipopolysaccharide-protein complexes, but it seemed that only lactocin LP27 retained activity when dissociated into smaller units. Lactocin LP27 is rather an anomalous bacteriocin-like agent in that it is apparently only bacteriostatic in its action against sensitive organisms (301).

Listeria

In the course of investigations of bacteriophage production by *L. monocytogenes* it was reported that certain strains produced, after irradiation, substances that were similar to the colicins (269). Subsequently, Hamon and Peron undertook a more extensive examination of these bacteriocins which they named monocins (105). They found that 25 of 51 strains tested were monocinogenic (106). Groups A and B were differentiated on the basis of action against resistant mutants, and nine types were identified within group A according to differences in activity spectra and temperature sensitivity (108). Characteristics of these bacteriocins included high antigenicity, insensitivity to trypsin, inducibility by ultraviolet light, and relative heat lability (106, 108). In another study, Tubylewicz (290) found that four of eight strains of *L. monocytogenes* produced bacteriocins that were separable into two distinct types on the basis of activity spectra and temperature sensitivity. More recently, Hamon and Peron have reported that due to the sedimentability, the lytic mode of action, and serological cross-reactivity with phages, the monocins are most likely defective bacteriophage particles (109). This contention is supported by electron microscopy studies of one monocin preparation that predominantly consisted of what appeared to be phage tail assemblies (25, 26).

Micrococcus

Although there do not appear to be any reports in the literature dealing specifically with bacteriocin production by micrococci, comment seems justifiable regarding a substance that has some of the characteristics of bacteriocins and may have been referred to as a bacteriocin by some investigators.

Micrococcus sp. strain NCTC 7218 produces an antibiotic, micrococcin (73, 268), which is a peptide of molecular weight 2,000 (115), mainly active on gram-positive bacteria (267), and insensitive to trypsin (267). Recent studies indicate that micrococcin may act by blocking the binding of aminoacyl-transfer ribonucleic acid to the ribosomal A site (47).

Mycobacterium

The first report of bacteriocin-like antagonism by mycobacteria was that by Mora and Eisenstark (Bacteriol. Proc., p. 81–82, 1958). As was the case in so many initial observations of bacteriocinogenicity, this arose during an investigation of lysogenicity in a collection of *Mycobacterium* cultures. The authors were able to distinguish the inhibitory effect from that attributable to phage or to pH change, and showed that the inhibitors were dialyzable through cellophane and were heat labile. Similar findings were reported by Adámek and associates (3) in a study of rapidly growing mycobacteria. By contrast, Imaeda and Rieber (132) showed that following treatment with mitomycin C, a mutant of *M. tuberculosis* (BCG) produced phage-like particles having the characteristics of bacteriocins. More recently, Takeya and Tokiwa have found that bacteriocin-like activity of mycobacteria cultures may have application for purposes of both classification (279) and typing (280). As a group, the bacteriocins of the mycobacteria have been studied only superficially and little is known of the characteristics or significance of these substances.

Sarcina

Trust (289) reported that a strain of *Sarcina* spp. produced a diffusible inhibitor when grown on solid media. The inhibitor was thermostable, resistant to proteolytic enzymes, and nondialyzable through cellophane membrane.

Staphylococcus

Observations of the antagonistic action of some staphylococci against other staphylococci and related bacteria have been very frequently reported in the literature. Many of the early reports have been summarized by Florey and associates (73). Staphylococci are now known to produce a wide variety of inhibitory substances including phages, bacteriolytic enzymes, and antibiotics and earlier investigators were unable to identify precisely the nature of the observed inhibitory effects.

Fredericq (78) remarked that inhibitory effects associated with staphylococci resembled those attributable to colicins in *E. coli* and first used the term staphylococcin. Fredericq distinguished a number of different types of staphylococcins on the basis of differences in inhibitory spectra (78). Similar findings have been documented by Halbert and associates (100), Ivanov (135), Pulverer and Sieg (228), and Jetten and Vogels (150). Jetten and Vogels examined antagonistic substances produced by a collection of *S. aureus* strains and were able to differentiate five groups according to their properties and cross-resistance patterns. One group consisted of lytic enzymes and the other four groups consisted of staphylococcins (150).

Several of the inhibitory substances produced by staphylococci were shown to have low molecular weights and were referred to as antibiotics

(129, 155, 182). Others that have been called antibiotics (85, 303) seem not unlike some of the bacteriocins. There remain a few inhibitors that are more justifiably categorized as bacteriocins including aureocin (202), staphylococcin 462 (101, 102), staphylococcin 414 (84), and staphylococcin C55 (48, 49, 51–53, 55–58).

It has been observed that bacteriocinogenicity appears to occur with a particularly high frequency in *S. aureus* strains of phage group II, particularly type 71 (48, 51, 55, 71, 150, 173, 218, 226). The association of phage type 71 *S. aureus* strains with inhibitory activity was reported first by Parker and associates in England (217–219). The inhibitor has since been studied extensively in Minnesota where its characterization as a bacteriocin has been achieved (44, 48, 49, 51–53, 55–58).

Two of the bacteriocins produced by strains of *S. epidermidis* have also been studied in considerable depth recently. Jetten and Vogels have investigated staphylococcin 1580 (148, 149, 151–153) and Lachowicz has worked with staphylococcin A-1262a (173–177).

Streptococcus

In 1949 Sherwood and associates (255) reported the production of antibiotic substances called streptostasins by a number of hemolytic streptococci, including strains belonging to each of the nine Lancefield groups A to K (there is no group I).

Surveys of collections of group A streptococci have indicated a high frequency of inhibitory strains, assumed to be producing bacteriocins (164, 172, 213, 227). In none of these studies was isolation and characterization of the inhibitory agent achieved and adequate precautions were not taken to exclude the occurrence of inhibition due to hydrogen peroxide formation. The latter has been shown to accumulate in inhibitory concentrations in some streptococcal cultures (188). Streptococcin A-FF22 is the only group A streptococcus bacteriocin that has been isolated and studied in depth (271, 276, 277).

Although there have been several observations of bacteriocin-like antagonism associated with group B streptococci (172, 227, 255), only recently have attempts been made to study these bacteriocins intensively (166, 270, 299). One of the latter studies reported an incidence of inhibitory strains in excess of 60% (299) but no isolation and characterization of the inhibitors was documented. The other studies revealed that less than 7% of group B streptococci were bacteriocinogenic and demonstrated purification of one of the bacteriocins encountered (166, 270).

The production of a bacteriocin by group H *Streptococcus* strain Challis has been reported by Schlegel and Slade (248, 249). The bacteriocin was produced in culture concomitantly with (but was separable from) competence factor activity. The relationship of this bacteriocin to the extracellular lysin also produced by strain Challis (230, 231) is at present uncertain.

Bacteriocin production by group D streptococci was noted by Kjems (163) in a study of streptococcus phages. Subsequently, the widespread occurrence of bacteriocin-like antagonism associated with various species of streptococci belonging to this Lancefield group has been thoroughly documented (27, 35, 159, 224, 287, 297). Brock and associates (35) defined bacteriocin types 1 through 5 produced by group D streptococci on the basis of activity spectra, and of sensitivity to heat, chloroform, and proteolytic enzymes. The type 1 bacteriocin, which was produced by all strains of *S. faecalis* subsp. *zymogenes*, was shown probably to be identical with the hemolysin produced by these strains (34). Subsequent data have supported this contention and have provided additional data on the nature of the lysin (14, 92, 143) and the mechanism of host cell resistance to this agent (61). Bottone and associates (21, 22) have shown that a quite different bacteriocin is produced by one strain of *S. faecalis* subsp. *zymogenes* that did not fit any of the five types described by Brock and Davie. Recently two bacteriocins produced by a strain of *S. faecium* have been purified and examined by Kramer and Brandis (167).

Group N streptococci have been shown to produce two substances, diplococcin (73, 216) and nisin (193), that have some characteristics of bacteriocins. The basic protein antibiotic nisin has been studied extensively and is similar to the bacteriocin streptococcin A-FF22 in molecular size (42), genetic determinants (165), activity spectrum (90, 193), and production characteristics and physicochemical properties (11, 311). A role for nisin in the regulation of cell growth has been suggested (131).

Alpha-hemolytic streptococci that have been shown to produce inhibitory substances include *S. pneumoniae* (73, 199), viridans streptococci (54, 87, 222), *S. mutans* (104, 236, 237), and other "oral streptococci" (160, 162). Some of these inhibitory substances were not well characterized, partly because of difficulty in isolating sufficient quantities of the active inhibitors. Recently, Dajani et al. (54) isolated bacteriocins from *S. sanguis* and *S. mitis* strains. These bacteriocins (termed viridins) were shown to have a broad spectrum of activity, including

inhibition of several gram-negative organisms. One such bacteriocin (viridin B) has been partially purified and characterized (54).

The possibility of inhibition due to hydrogen peroxide by some alpha-hemolytic streptococci cannot be discounted. In some studies it has been demonstrated that bactericidal concentrations of hydrogen peroxide may accumulate in cultures of *S. sanguis* (126) and *S. mitis* (179).

Streptomyces

Representatives of this genus produce a wide variety of classical antibiotic substances and it is outside of the range of this review to discuss these. Here, reference is made only to the report by Roelants and Naudts (235) of a bacteriocin-like substance produced by *S. virginiae*, which appeared to differ significantly from the classical antibiotics. Bacteriocin-like properties included inducibility by ultraviolet irradiation, nonlytic action that was restricted to the streptomycetes, nondialyzable protein nature, and specific adsorption to sensitive cells.

CONCLUSION

Studies of bacteriocins and bacteriocin-like substances of gram-positive organisms have broadened the horizons of our knowledge in this area and have raised questions about the definition, the classification, the spectra of activity, the mode of action, the genetic basis, and the biological significance of these antagonistic agents. Earlier concepts, largely based on the studies of colicins, may need to be modified as workers examining the bacteriocins of gram-positive species move more into the field of molecular biology.

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