A phylogenetic approach to assessing the targets of microbial warfare

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Abstract

Bacteriocins are the most abundant and diverse defense systems in bacteria. As a result of the specific mechanisms of bacteriocin recognition and translocation into the target cell it is assumed that these toxins mediate intra-specific or population-level interactions. However, no published studies specifically address this question. We present here a survey of bacteriocin production in a collection of enteric bacteria isolated from wild mammals in Australia. A subset of the bacteriocin-producing strains was assayed for the ability to kill a broad range of enteric bacteria from the same bacterial collection. A novel method of estimating killing breadth was developed and used to compare the surveyed bacteriocins in terms of the phylogenetic range over which they kill. The most striking result is that although bacteriocin-producers kill members of their own species most frequently, some kill phylogenetically distant taxa more frequently than they kill closer relatives. This study calls into question the role these toxins play in natural populations. A significant number of bacteriocins are highly effective in killing inter-specific strains and thus bacteriocins may serve to mediate bacterial community interactions.

Introduction

Microbes produce an extraordinary array of defense systems. These include broad-spectrum classical antibiotics, metabolic by-products such as the lactic acids produced by lactobacilli, lytic agents such as lysozymes, numerous types of protein exotoxins and bacteriocins. The microbial weapon of choice, as measured by abundance and diversity in natural populations, is the heterogeneous family of proteins known as bacteriocins (Riley & Gordon, 1995; Riley & Wertz, 2002).

Bacteriocins are loosely defined as biologically active protein moieties with a bacteriocidal mode of action (Tagg *et al.*, 1976; James *et al.*, 1991). They differ from traditional antibiotics in one critical way; they have a relatively narrow killing spectrum and are often only toxic to bacteria closely related to the producing strain.

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Bacteriocins have been found in all major lineages of Bacteria and, more recently, have been described as frequently produced by some lineages of Archaea (Torreblanca *et al.*, 1994). Within a species tens or even hundreds of different kinds of bacteriocins are produced (James *et al.*, 1991; Riley & Gordon, 1992). According to Klaenhammer (1988), 99% of all bacteria make at least one bacteriocin and the only reason more have not been found is that few researchers have looked for them.

Without question, bacteriocins serve some function in microbial communities. This statement follows from detection of bacteriocin production in all surveyed lineages of prokaryotes. Equally compelling is the inference of strong positive selection acting on enteric bacteriocins (Tan & Riley, 1996; Riley, 1998). Finally, there is a well-developed body of theory and empirical data that details the potential role bacteriocins play in maintaining microbial diversity at the population and community levels (Chao & Levin, 1981; Frank, 1994; Gordon & Riley, 1999; Czárán *et al.*, 2002; Kerr *et al.*, 2002). Such empirical observations and theoretical results argue that these toxins play a critical role in

mediating microbial interactions. What remains in question is what, precisely, that ecological role is. Bacteriocins may serve as anti-competitors enabling the invasion of a strain or a species into an established microbial population or community. They may also play a defensive role and act to prohibit the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells. An additional role has recently been proposed for bacteriocins produced by gram-positive bacteria, that of regulating quorum sensing (Miller & Bassler, 2001).

Bacteriocins are assumed to mediate within-species or population-level interactions. This assumption is based upon the narrow killing range reported for most bacteriocins (James *et al.*, 1991). However, a thorough search of the bacteriocin literature reveals no studies specifically designed to assess the killing breadth of bacteriocins. This assumption has resulted in the focus of all but the most recent theoretical investigations on bacteriocins mediating population, rather than community, level interactions (Chao & Levin, 1981; Frank, 1994; Gordon & Riley, 1999; Czárán *et al.*, 2002; Kerr *et al.*, 2002).

The focus of the current work is to explore the phylogenetic range over which bacteriocins kill. To this end, 396 strains of the Enterobacteriaceae were chosen from a large collection of enteric bacteria isolated from Australian mammals (Gordon & Fitzgibbon, 1999). This phylogenetically diverse sample of bacteria was screened for bacteriocin production. The phylogenetic range over which a subset of these bacteriocin-producing strains kill other enteric bacteria from the same collection was measured. This measure involved employing a molecular phylogeny of enteric bacteria (J.E. Wertz, C.M. Valletta-Goldstone, D.M. Gordon & M.A. Riley, unpublished data) and mapping onto that phylogeny the killing breadth of each bacteriocin. The branch lengths over which each bacteriocin killed were summed to produce a quantitative assay of killing breadth. These data represent the first step in our effort to understand the ecological role served by this potent arsenal of biological weapons.

Materials and methods

Bacterial strains

The strains used in this study are a subset of the collection isolated from the feces of nondomesticated Australian mammals (Gordon & Lee, 1999). The procedure used to isolate and identify the strains is described by Gordon & Fitzgibbon (1999). We chose 396 strains from the collection and screened them for bacteriocin production. The strains were chosen based on our desire to obtain a reasonable sample size of bacteriocin producers from each of a number of different enteric taxa. The details of strain and host information is available in Gordon & Fitzgibbon (1999).

The 396 strains, grouped by species, are summarized in Table 1. In the initial survey of bacteriocin production all strains served as both indicator and potential bacteriocin producer strains. A subset of these strains was employed in the subsequent bacteriocin killing breadth survey. This subset includes 36 bacteriocin producers (Table 1, column 2) and ~40 randomly chosen strains per species to serve as indicators (Table 1 last column) for a total of seven species (taxa in bold in Table 1).

Bacteriocin production screen

Bacteriocin lysates were prepared for each of the 396 strains summarized in Table 1. A standard bacteriocin induction protocol was employed (Pugsley & Oudega, 1987), which involved overnight growth in a 25 mL flask with 10 mL of Luria Broth (Sigma, St. Louis, MO, USA) incubated at 37 °C, and shaken at 220 rpm. One hundred microlitres of the overnight culture was transferred to a fresh 25 mL flask with 5 mL of Luria Broth (Sigma). The cultures were incubated at 37 °C, and shaken at 220 rpm. After approximately 1–2 h an OD of 0.2 was reached and mitomycin C (0.05 μg mL^{-1}) (Sigma) was added to the culture. Mitomycin C induces expression of enteric bacteriocin gene clusters (Pugsley & Oudega, 1987). Incubation at 37 °C with shaking continued for 2–5 h. Following this incubation, 100 μL of chloroform (Fisher, Pittsburgh, PA, USA) was added to 1 mL of each culture. The eppendorf tubes were vortexed for 15 s and spun in a microcentrifuge at 14 000 rpm for 10 min. Following centrifugation, the supernatant was transferred to a clean tube and stored at 4 °C. Indicator lawns were prepared by pouring 3.5 mL top agar mixed with 50 μL of cells (at 1×10^9 cells mL^{-1}) onto a LB agar plate. After the lawns solidified, 2 μl of each lysate was spotted twice on the lawn in a grid matrix. In the bacteriocin production survey, lysates were spotted onto indicator strains from the same species. All strains were assayed in duplicate (two separate plates per indicator lawn) with one replication per plate (two lysate spots per plate).

The first round of screening revealed 190 potential bacteriocin-producing strains (see Table 1). These putative producers were subjected to trypsin digestion, filtration, and freezing to distinguish between phage and bacteriocin production. Trypsin digestion was performed by adding 5 μL of trypsin (Sigma, 5 mg mL^{-1}) to 50 μL lysate and incubating at room temperature for 30 min. Filtration was performed by adding 50 μL lysate to a Microcon 100 kDa Microconcentrator (Amicon, Billerica, MA, USA), which was spun in a microcentrifuge at 14 000 rpm for 15 min. Lysates were also frozen at −70 °C. Lysates treated by all three methods were spotted on sensitive lawns. The killing pattern resulting from the three treatments suggest if killing was because of phage or bacteriocin (Pugsley & Oudega, 1987). Trypsin digestion inhibits the activity of most bacteriocins, but does not inhibit most phage. Filtration will

Table 1 Bacteriocin production in enteric bacteria.

Species	No. of strains surveyed	No. of killers*	Bacteriocin producers†	No. of bacteriocin producers in killing breadth study	No. of indicator strains in killing breadth study‡
Escherichia coli§	**27**	**12 (44.4)**	**7 (25.9)**	**7**	**39**
Klebsiella pneumoniae	**54**	**27 (50.0)**	**10 (18.5)**	**7**	**40**
Klebsiella oxytoca	**42**	**22 (52.4)**	**4 (9.5)**	**4**	**40**
Klebsiella spp.	26	16 (61.5)	1 (3.9)	0	0
***Enterobacter* spp.**	**152**	**94 (61.8)**	**37 (24.3)**	**7**	**40**
Citrobacter freundii	29	1 (3.4)	1 (3.4)	0	40
***Serratia* spp.**	**24**	**8 (33.3)**	**4 (16.7)**	**4**	**24**
Hafnia alvei	**42**	**10 (24.0)**	**7 (16.7)**	**7**	**40**
Total	**396**	**190 (47.9)**	**71 (17.9)**	**36**	**263**

*Number of killer phenotypes detected.
†Number of killer phenotypes that conform to a bacteriocin-killing phenotype.
‡Number of indicator strains used in the bacteriocin killing breadth study.
§Taxa in the killing breadth study are indicated in bold.
Numbers in parantheses indicate percentages.

separate most phage from bacteriocin proteins. Freezing often destroys phage, but will not affect bacteriocins. Seventy-one putative bacteriocins with the appropriate combination of phenotypes were considered as confirmed bacteriocins in this study (Table 1).

Bacteriocin killing range screen

Thirty-six confirmed bacteriocin-producers were chosen for further study (Tables 1 and 2). Bacteriocin producer choice was determined by the desire to include at least four bacteriocins per species and six species in total. In addition, only those bacteriocins that produced large killing zones during the initial screen were included in the killing range screen. Thus, our screen was biased towards those bacteriocin-producers that yield high bacteriocin titres under a particular set of induction conditions. Prior studies have revealed a positive correlation between lysate titre and killing activity (Gordon *et al*., 1998). This subset of 36 bacteriocins was spotted onto ~40 randomly chosen indicator lawns for each of seven species (*Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Hafnia alvei*, *Serratia plymuthica*). Each bacteriocin was spotted twice on each lawn and each lawn was replicated on two plates.

Enteric molecular phylogeny

A molecular phylogeny for seven taxa of enteric bacteria (*C. freundii*, *E. cloacae*, *E. coli*, *H. alvei*, *K. oxytoca*, *K. pneumoniae*, and *S. plymuthica*) was inferred from the concatenated partial sequences of five housekeeping genes (*gapA*, *groEL*, *gyrA*, *ompA*, and *pgi*) and the 16 s rRNA gene. Multiple strains of each species were used from the same culture collection utilized for the rest of this study except for sequences from*V. cholerae* biotype *El Tor*, which were obtained from GenBank and used as the outgroup for rooting the tree. The data set consisted of 4203 aligned nucleotides from 24 taxa. Sequences were aligned using the clustalW algorithm (Thompson *et al*., 1994) in Megalign version 4.05 (Dnastar, Inc.) and maximum likelihood trees were inferred using PAUP version 4.0b8 (Swofford, 1997). Optimized parameters for the heuristic algorithm used for building maximum likelihood trees in PAUP were generated by the MODELTEST program version 3.06 (Posada & Crandall, 1998). Statistical support of the branch points was tested by performing 500 maximum likelihood bootstrap replications using PAUP version 4.0b8 and by using the program MrBayes version 2.01 (Huelsenbeck & Ronquist, 2001) to generate 50 000 trees, of which the first 5000 trees were discarded as 'burnin', and a majority rule consensus tree was constructed from the remaining 45 000 trees. Both methods produced phylogenies in which all of the species formed monophyletic groups (bootstrap values were 100% for each species group), and the topologies of the two trees were identical at the species level and above.

Estimate of bacteriocin killing range

The molecular phylogeny inferred by J.E. Wertz, *et al.* (unpublished data) was employed in the estimation of bacteriocin killing range (Fig. 1). This phylogeny represents a composite maximum likelihood tree made by combining all DNA sequence data from six genes sequenced in each of approximately five strains from each of seven enteric taxa (J.E. Wertz *et al*., unpublished data). The bacteriocin killing data were mapped onto the enteric phylogeny and the sum of the branch lengths connecting the bacteriocin producer strain with those taxa it kills was determined. The sum of the branch lengths provides an estimate of phylogenetic killing breadth. A weighting system was introduced based upon the number of strains within each taxon killed by a bacteriocin producer. The

Table 2 Bacteriocin killing screen.

	Bacteriocin producers																																				
Bacteriocin indicators	EC1	EC2	EC3	EC4	EC5	EC6	EC7	EB1	EB2	EB3	EB4	EB5	EB6	EB7	KO1	KO2	KO3	KO4	KP1	KP2	KP3	KP4	KP5	KP6	KP7	SP1	SP2	SP3	SP4	HA1	HA2	HA3	HA4	HA5	HA6	HA7
EC(39)*	10†	15	5	8	3	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	0	0	0	0
EB(40)	0	0	0	0	0	0	0	0	10	8	3	5	25	3	0	0	0	0	5	5	3	3	10	10	0	0	0	0	0	0	0	0	0	0	0	0
HA(40)	0	0	0	3	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	20	18	30	30	30	30
KP(40)	0	0	0	0	0	0	0	0	0	0	0	0	3	3	0	13	30	3	25	10	13	30	3	3	3	0	0	0	0	0	0	0	0	0	0	0
KO(40)	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	50	38	28	3	5	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP(24)	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	13	13	13	8	0	0	0	0	0	0	0
CF(40)	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	3	3	0	0	0	0	0	0	0	0	0	0	0	0
% Average killing/lysate	1	2	1	2	1	0.4	3	0	1	1	0.4	1	8	1	0	9	10	4	5	3	3	5	2	2	0.4	2	2	2	2	1	3	3	4	4	4	4
% Average killing/taxa	1.5							2							6				3							2				3						

*Indicator lawn species, number of isolates per species is given in parenthesis.
†Percent killing.
Bacteriocin producer abbreviations: EC = *Escherichia coli*, EB = *Enterobacter cloacae*, KO = *Klebsiella oxytoca*, KP = *Klebsiella pneumoniae*, SP = *Serratia plymuthica*, HA = *Hafnia alvei*.

length of the terminal branch connecting a producer strain and a particular taxon killed by that producer was multiplied by the frequency of strains killed in that taxon. Thus, a lysate that kills many strains within a taxon has a greater phylogenetic killing breadth than a lysate that kills only rarely within a target taxon.

Results

Bacteriocin production in natural strains of enteric bacteria

A total of 396 strains from six taxa of enteric bacteria were assayed for bacteriocin production using a standard method of bacteriocin induction (Table 1). The taxa have varying levels of bacteriocin production that ranges from 3.4 to 26% (Table 1). This range is similar to the levels of bacteriocin production noted from prior surveys of clinical and natural strains of these same taxa (Dhillon & Dhillon, 1981; Bradley, 1991; Riley & Gordon, 1992; Smarda, 1992; Chakraborty & Nag, 1998; Gordon *et al.*, 1998; Murinda *et al.*, 1998; Smarda & Smajs, 1998; Smarda & Obdrzalek, 2001).

A subset of 36 bacteriocin producers from six enteric taxa was chosen for further study (Tables 1 and 2). Producer choice was dictated by our desire to include a sample of multiple bacteriocins from each of several taxa and to employ only those bacteriocins induced at high levels using standard conditions. This subset of bacteriocins was assayed for the ability to kill a random sample of indicator strains from the same bacterial collection from each of seven enteric species (Table 2). In this study we have assumed that toxins that fulfill the classic phenotypic definition of a bacteriocin are, indeed, bacteriocins. It is possible that some of the toxins we have included in this study are novel proteins that are not bacteriocins in the traditional sense. However, we have cloned and sequenced 16 of 36 bacteriocins employed here and each is a colicin-like bacteriocin based upon inferred amino acid sequences.

This subset of bacteriocin producers kills 0–10% of the 263 indicator strains (Table 2). Two bacteriocin producers fail to kill any of the indicator strains (KO1 and EB1). Seventeen bacteriocin producers kill indicator strains from only one species (EC1–3, 6–7; KP7; EB 2–5 and HA1–7) whereas the remaining 17 kill more broadly (EC4, 5; KO2–4, KP1–6; EB6, 7, SP1–4). On average, bacteriocins are most effective at killing indicator strains from the producer's own species. For example, although some *E. coli* bacteriocin producers kill strains from three species in this survey, *E. coli* indicator strains are killed at a three to sevenfold higher frequency. It is notable that bacteriocins produced by *H. alvei* only kill strains from their own species. Table 2 provides a summary of how many indicator strains were killed per bacteriocin producer, as well as a pooled estimate of percentage killing per taxa for each producer. The levels of killing per taxa

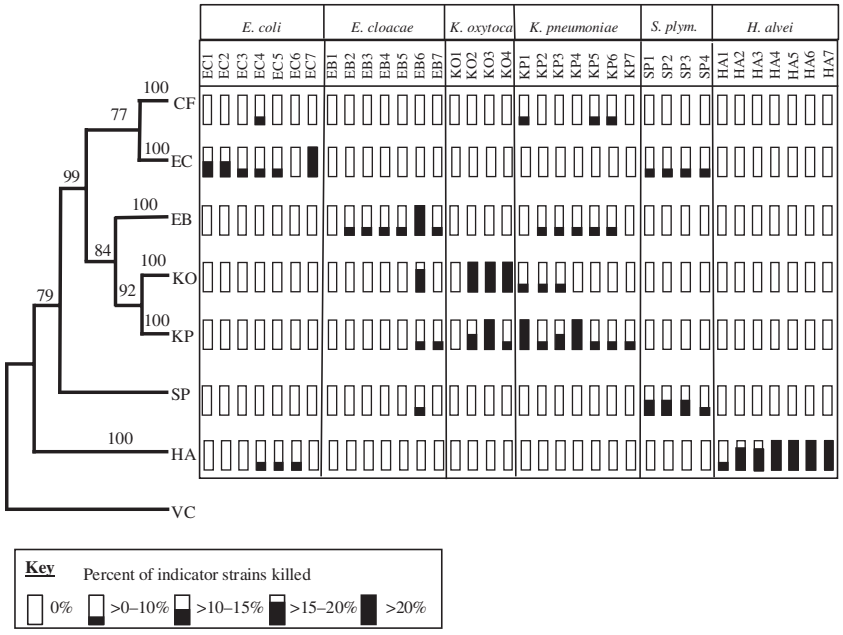

Fig. 1 Enteric bacteriocin phylogenetic killing range. The frequency of bacteriocin killing within each of seven enteric taxa is mapped onto a composite molecular phylogeny of enteric bacteria (J.E. Wertz *et al.*, unpublished data). The bacteriocins assayed for killing breadth are indicated across the top (EC = *Escherichia coli*, KO = *Klebsiella oxytoca*, KP = *K. pneumoniae*, EB = *Enterobacter cloacae*, HA = *Hafnia alvei*, SP = *Serratia plymuthica.)* Each column provides the frequency of killing for each bacteriocin assayed against ~40 indicator strains for each taxa in the molecular phylogeny.

range from 1.5 to 6% (Dhillon & Dhillon, 1981; Bradley, 1991; Riley & Gordon, 1992; Smarda, 1992; Chakraborty & Nag, 1998; Gordon *et al.*, 1998; Murinda *et al.*, 1998; Smarda & Smajs, 1998; Smarda & Obdrzalek, 2001).

Bacteriocin killing frequencies were mapped onto a molecular phylogeny of enteric bacteria (Fig. 1). The density of killing observed across the diagonal of the figure (producers killing indicator strains within their own taxon) suggests that producers tend to kill members of their own taxon most frequently. However, there are numerous exceptions. The EC4 bacteriocin kills strains of *E. coli*, *C. freundii* and *H. alvei*. *E. coli*, the producing species, is more closely related to *E. cloacae*, *K. oxytoca* and *K. pneumoniae*, which EC4 does not kill, than it is to *H. alvei*, which EC4 does kill. The KP6 bacteriocin kills strains of *K. pneumoniae*, *E. cloacae* and *C. freundii*. The latter two species are more distantly related to the producer strain (*K. pneumoniae*) than is the species *K. oxytoca*, which is not killed by KP6. Bacteriocin killing breadth does not follow a simply phylogeny-based rule, which would predict that bacteriocin-killing frequencies should decrease with increased phylogenetic distance.

To provide a quantitative assay of phylogenetic killing breadth, the phylogenetic range over which each bacteriocin kills was estimated by summing the branch lengths in the tree that join the producer taxa with those taxa killed by that bacteriocin producer (Table 3). In most cases the producer kills indicator strains from its own taxon (all save EB1 and KO1). A branch length of 0.01 is added to account for this within taxon killing in the phylogenetic killing breadth estimate. Killing breadth ranges from 0 to 0.34 for this sample of producer strains with a mean of 0.10.

This method of killing breadth estimation is biased, because it ignores the frequency with which a bacteriocin kills within a taxon. To account for this observed difference in killing frequency, a weighting factor was applied, based upon killing frequency within a taxon (Table 3). For example, the bacteriocin produced by EC4 kills *E. coli*, *C. freundii* and *H. alvei* indicator strains. A branch length of 0.01 is added for the *E. coli*-based killing and it is multiplied by 0.08 to account for the fact that 8% of the *E. coli* indicator strains were killed by this bacteriocin. A branch length of 0.049 is added for the *C. freundii*-based killing and it is multiplied by 0.03 to account for the fact that 3% of the *C. freundii* indicator strains were killed by this bacteriocin. Connecting branch lengths of 0.013, 0.057 and 0.035 are added to span the phylogenetic distance between *E. coli* and *H. alvei*. Finally a branch length of 0.072 is added for the *H. alvei*-based killing and is multiplied by 0.03 to account for the fact that 3% of the *H. alvei* indicator strains were killed by this bacteriocin. The killing percentages are also weighted by the number of sensitive strains each producer was screened against. The phylogenetic killing breadth for this sample of indicators and producers ranges from 0 to 0.78, with a mean of 0.20. Producers with a weighted killing breadth over 0.16 are able to effectively kill indicator strains from two or more taxa.

Discussion

Bacteriocin production in enteric bacteria

There are numerous surveys of bacteriocin production in bacteria. However, it is difficult to compare data from

Table 3 Phylogenetic killing breadth.

Bacteriocin producers	Killing breadth*	Weighted killing breadth†
EC1	0.01	0.04
EC2	0.01	0.06
EC3	0.01	0.02
EC4	0.29	0.34
EC5	0.24	0.26
EC6	0.23	0.25
EC7	0.01	0.08
$\bar{x}$ EC =	0.12	0.15
EB1	0	0
EB2	0.01	0.04
EB3	0.01	0.03
EB4	0.01	0.01
EB5	0.01	0.02
EB6	0.34	0.73
EB7	0.14	0.16
$\bar{x}$ EB =	0.07	0.14
KO1	0	0
KO2	0.09	0.49
KO3	0.09	0.78
KO4	0.09	0.2
$\bar{x}$ KO =	0.07	0.37
KP1	0.19	0.42
KP2	0.18	0.31
KP3	0.18	0.24
KP4	0.14	0.22
KP5	0.22	0.42
KP6	0.22	0.42
KP7	0.01	0.01
$\bar{x}$ KP =	0.16	0.29
SP1	0.22	0.27
SP2	0.22	0.27
SP3	0.22	0.27
SP4	0.22	0.25
$\bar{x}$ SP =	0.22	0.27
HA1	0.01	0.02
HA2	0.01	0.08
HA3	0.01	0.07
HA4	0.01	0.12
HA5	0.01	0.12
HA6	0.01	0.12
HA7	0.01	0.12
$\bar{x}$ HA =	0.01	0.09
$\bar{x}$ total =	0.1	0.2

*The killing breadth is a quantitative measure of the phylogenetic range over which each bacteriocin kills. It is estimated by summing the branch lengths in the phylogeny (Fig. 1) that join the producer taxa with those taxa killed by that bacteriocin producer. 0.01 is the default score for intraspecific killing.

†The weighted killing breadth is the phylogenetic breadth of killing for a species, (see *), weighted by the frequency of killing within each target species. See text for more complete definition.

these various studies, as the sampling strategies involved vary dramatically (Kageyama, 1975; Edmondson & Cooke, 1979; Sproat & Brown, 1979; Bauernfeind *et al.*, 1981; Rodriguez-Valera *et al.*, 1982; Scott & Mahony, 1982; Watson, 1985; James & Tagg, 1986; Dodatko *et al.*, 1989; Tagg, 1991; Podschun & Ullmann, 1993, 1996). Further, the collections are usually comprised of clinical strains, which represent a biased sample of bacterial diversity (reviewed in Gordon & Fitzgibbon, 1999; Gordon & Lee, 1999; Gordon, 2001). There has never been a systematic survey of bacteriocin production for a group of related bacterial species isolated in the same manner from the same hosts at the same time. The collection of enteric bacteria from which our sample of strains was drawn represents the first, and only, collection to provide a large sample of bacteria isolated from numerous enteric species from the same mammalian hosts at the same time (Gordon & Fitzgibbon, 1999; Gordon & Lee, 1999).

Several surveys of bacteriocin production in enteric bacteria reveal that, on average, 10–50% of the strains sampled produce bacteriocins (Dhillon & Dhillon, 1981; Bradley, 1991; Riley & Gordon, 1992; Smarda, 1992; Chakraborty & Nag, 1998; Gordon *et al.*, 1998; Murinda *et al.*, 1998; Smarda & Smajs, 1998; Smarda & Obdrzalek, 2001). The present study confirms these earlier estimates of production frequencies and reinforces the recent suggestion that bacteriocins may be the primary defense mechanism in bacteria (Riley & Wertz, 2002).

Induction of bacteriocin production in gram-negative bacteria is usually mediated by the SOS regulon (Pugsley & Oudega, 1987). Several induction protocols that differ in growth media, incubation temperature and other details have been developed and refined for different enteric species (Atkinson, 1973; Buffenmyer *et al.*, 1976; Chhibber & Vadehra, 1989; Daw *et al.*, 1992; Podschun & Ullmann, 1993). Several of these protocols were tested against a sub sample of the strains summarized in Table 1 (data not provided). The protocol developed by Pugsley (Pugsley & Oudega, 1987) resulted in detection of the largest number of bacteriocin-producers and we employed this protocol in our final survey, thus biasing our screen to detect bacteriocins encoded by genes induced by the SOS regulon and the Pugsley protocol. It is likely that our survey under-represents the actual frequency of bacteriocin production in natural strains of enteric bacteria – because of both the single induction method we employed and the sensitivity of our screen. There are almost certainly strains in our sample that carry bacteriocin gene clusters, which are induced in some novel fashion or produce levels of bacteriocin undetectable by our screening method. Future efforts will be directed at employing a PCR-based approach to confirm and extend this phenotype-based survey (D. Gordon, unpublished; J.E. Wertz & M.A. Riley, unpublished).

Phylogenetic killing breadth of enteric bacteriocins

It is assumed that bacteriocins play a role in mediating population level, or intra-specific, microbial dynamics. This assumption is based upon the targeting and translocation mechanisms employed by certain characterized bacteriocins (James *et al*., 1991). In enteric bacteria, bacteriocins target cells by recognizing and binding to a specific cell surface receptor, such as the BtuB receptor (James *et al*., 1996). The bacteriocin is then transported into the cell by one of a number of specific translocation systems, such as the Ton and Tol pathways (James *et al*., 1996). Given the specificity of these target and translocation systems, it is assumed that bacteriocins recognize only a small number of targets, those that share similar receptor and translocation systems. It is reasonable to assume that more distantly related taxa will have more divergent, or even completely different, receptor and translocation systems. This assumption, that bacteriocins predominately kill strains from the same species, has served as the basis for much of the experimental and theoretical work exploring the role of bacteriocins in mediating population dynamics (Frank, 1994; Gordon & Riley, 1999).

An exploration of the literature reveals no published surveys of bacteriocin phylogenetic killing breadth. To produce such an estimate, we took advantage of a recently determined molecular phylogeny of enteric bacteria (J.E. Wertz *et al*., unpublished data). The bacterial strains used to determine this enteric phylogeny are from the same bacterial collection as those employed in this study (Gordon & Fitzgibbon, 1999; Gordon & Lee, 1999). The frequency of killing within each taxon for each bacteriocin was mapped onto the enteric phylogeny (Fig. 1 and Table 2). These data reveal that bacteriocins usually kill members of their own species. In addition, a surprisingly high level of interspecific killing was observed with almost half of the bacteriocins killing in more than one taxon. Several of the bacteriocins killed strains from as many of four taxa.

The most significant result of this study is the observation that certain enteric bacteriocins kill across broad phylogenetic distances, whereas others kill only the producer strains' species or its close relatives (Table 3 and Fig. 1). Further, the relationship between killing ability and phylogenetic distance is nonlinear. In other words, some bacteriocin producers kill distantly related bacteria but not their closest relatives. This nonlinear relationship is seen in Fig. 1, in which 18 of 36 columns indicate killing outside of the producer strains own species (off the diagonal).

The observation of a broad killing range for numerous enteric bacteriocins requires that the ecological role proposed for bacteriocins be reconsidered. It may well be that they serve a broader, community level role than has been envisioned to date. This finding complements recent theoretical work, which suggests that bacteriocins (and other microbial defense systems) may be responsible for maintaining much of the extraordinary diversity of microbes observed in nature (Czárán *et al*., 2002), (Kerr *et al*., 2002). Determining how bacteriocins and other antimicrobials accomplish this feat awaits the development of more sophisticated experimental systems and mathematical models that take into account this new community-level role bacteriocins seem poised to play.

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