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Full Length Research Paper

Comparative analysis of antibacterial activities of *Xenorhabdus* species on related and non-related bacteria *in vivo*

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Insect-nematode-bacterium mutualistic associations provide attractive systems for discovery of inter kingdom signal compounds and antibiotics. A better understanding of the biological meaning of the inter-specific diversity of compounds with antimicrobial activity of the *Steinernema*-symbiont *Xenorhabdus* bacteria may provide options for simultaneous applications in pathogen control. Anti-bacterial activities of representative strains of *Xenorhabdus budapestensis, Xenorhabdus szentirmaii, Xenorhabdus innexi, Xenorhabdus ehlersii*, *Xenorhabdus nematophila, Xenorhabdus bovienii* and *Xenorhabdus cabanillassii* were tested on non-related (*Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*) bacteria and on each other by previously published bioassays. All active compounds were adsorbed by Amberlite^{R} XAD1180. Chemical and thermal stability of antibacterial factors were determined. Antibiotic factors produced by different *Xenorhabdus* species against each other differ from those used against other competing bacterial genera. Anti-*Xenorhabdus* activity of the cell-free medium and sensitivity of the cells of other *Xenorhabdus* strains negatively correlated in *X. innexi* and *X. bovienii*. Some activity remained unchanged during high pressure and 121°C for 10 min. The first comparative analysis of the intraspecific antibacterial activities of *Xenorhabdus* species demonstrated that some *Xenorhabdus* species with strong antibacterial activity could be co-cultured and they might be used simultaneously for pathogen control.

Key words: *Xenorhabdus*, autoclaveable antimicrobials, intra-generic, cross-tolerance.

INTRODUCTION

The entomopathogenic nematode / bacterium (EPN/EPB, Steinernema / *Xenorhabdus* and *Heterorhabditis* / *Photorhabdus*) symbiotic associations (Goodrich-Blair and Clarke, 2007) are potential tools for biological control of insect pests (Gaugler, 2002; ffrench-Constant et al., 2007) and microbial pathogens (Böszörményi et al., 2009) of agricultural importance. Mechanistic details of

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the *Steinernema carpocapsae* / *Xenorhabdus nematophila* symbiosis have been clarified (Goodrich-Blair, 2007). The scientific impact of studying this system is especially useful at this time, since the genomes of *X. nematophila* and *Xenorhabdus bovienii* have already been sequenced (http://www.genoscope.cns.fr/agc/mage).

Insect-nematode-bacterium tripartite associations such as those involving *Xenorhabdus* species provide attractive systems for both discoveries of new natural products, identification of novel compounds involved in inter kingdom signaling and antibiotics (Park et al., 2009). The evolution of the entomopathogenic *Xenorhabdus* bacteria has resulted in a broad, inter-specific, diversity of compounds with antimicrobial activity. These antagonize related, or non-related, competitors in the insect cadaver in the soil (Sicard et al., 2006). To compete successfully with invaders, EPB species produce several water-soluble and non-polar compounds with antibiotic activity (Paul et al., 1981; McInerney et al., 1991a; Sztaricskai et al., 1992; Sundar and Chang, 1993; Webster et al., 1996, 2002; Li, Hu and Webster, 1998). The water-soluble peptide antimicrobial compounds xenocoumacin 1 (Xcn1) and 2 (Xcn2), both of which are hybrids of amino and carboxylic acid moieties, are the major antibiotics produced in broth culture by *X. nematophila* strain all (McInerney et al., 1991a). Both Xcn1 and Xcn2 were also shown to be produced in the haemocoel of *Xenorhabdus*-infected insect cadavers (Maxwell et al., 1994). Xcn1 is active against gram-positive and gram-negative bacteria and several fungal species, while Xcn2 is less active against the bacteria and inactive against the fungal species examined (McInerney et al., 1991b). Recent molecular genetic analysis has identified a 14 gene complex involved in the biosynthetic of Xcn1 and conversion to Xcn2 (Park et al., 2009). In contrast, nothing has been published about a systematic analysis of anti-*Xenorhabdus* activities.

Despite promising results and patents, published information on the commercial use of *Xenorhabdus* antibiotics has not been realized. Interestingly, the antibacterial activities of the complete cell free media are much stronger (Brachmann et al., 2006) than any of the isolated, identified or patented compounds (e.g. nematophin, Li et al., 1997). The antibiotically active, non-purified, cell-free liquid cultures, of *Xenorhabdus* strains are effective against a large spectrum of invaders, from bacteria through fungi to protozoa. Recently, Böszörményi et al. (2009) showed that secondary metabolite(s) produced by *Xenorhabdus budapestensis* effectively reduced fire blight indexes on apple trees greenhouse conditions and also exerted strong toxicity on both zoospores and cystospores of *Phytophthora nicotianae*. Autoclaveable and metallo-protease resistant compound(s) produced by *X. budapestensis* and *Xenorhabdus szentirmaii* were active against strains of three pathogenic *Leishmania* species (B. S. McGwire, The Ohio State University, personal communication). While there are advantages of using two or more *Xenorhabdus* strains / species simultaneously intra-specific / inter-generic competition must be considered. Different EPN/EPB complexes have been shown to invade the same insect. Interspecies competition involves bacteriocins and xenocins (Boemare et al., 1992) phage-derived bacteriocins (Thaler et al., 1995, 1997) and colicin E3 type killer proteins (Singh and Banerjee, 2008). Sicard et al. (2006), monitored experimental inter-specific competition between two EPN species, *S. carpocapsae* and *Steinernema scapterisci* and their respective EPB symbionts, *X. nematophila* and *X. innexi*, within an experimental insect-host (*Galleria mellonella*). The authors suggested *Xenorhabdus* not only provides *Steinernema* with a food source, but also gives them new abilities to deal with biotic parameters such as competitors. However, the simultaneous use of more than one *Xenorhabdus* is limited by their tolerance to each other.

The present *in vivo* analysis examined some possibilities for controlling pathogens through simultaneous application of different *Xenorhabdus* species and/or their antimicrobials. Herein we evaluated whether anti-*Xenorhabdus* activities use the same mechanism as that for non-related bacteria. Our hypothesis is that the mechanisms for competition between *Xenorhabdus* species are different from that of competition with non-related, gram-negative, bacteria. The antibacterial effects of seven *Xenorhabdus* species on non-related bacteria and on each other were compared. Since Furgani et al. (2008) compared antibacterial compounds from some of the same *Xenorhabdus* strains against gram-positive and gram-negative mastitis isolates; we used these 'target' bacteria as "controls" to test the general antibacterial activities of the *Xenorhabdus* species.

MATERIALS AND METHODS

Microorganisms and culture conditions

Culture conditions

Entomopathogenic bacteria were cultured on Luria broth (LB), Luria broth agar (LBA), Nutrient broth (NB) and Nutrient agar (NA), all obtained from BD Diagnostics, as described by Furgani et al. (2008) and Böszörményi et al. (2009). Mastitis isolates were maintained and handled as described by Furgani et al. (2008). Physiological buffered salt (PBS), also from BD Diagnostics, was used for serial dilutions of bacterial suspensions. The 2X LB liquid media contained double each component of LB and was used for diluting the cell-free media. Mastitis isolates were stored at 4°C and *Xenorhabdus* at room temperature.

Xenorhabdus strains

Several strains (*X. nematophila* ATTC 19061; *X. bovienii* NYH, *Xenorhabdus cabanillassii* RIO-HU; *X. budapestensis* DSM 16342; *X. szentirmaii* DSM 16338) used in this study, along with their16S rDNA Accession Numbers, country of isolation, and the source of the *Steinernema* hosts associated with the bacteria, were

listed in Furgani et al. (2008). The DSM type strains of *X. innexi* (ABJ10292), (the natural symbiont of *S. scapterisci* from Uruguay) and *Xenorhabdus ehlersii* (ABJ10294), (the natural symbiont of *S. longicaudatum* from China) had been isolated in Budapest by Dr. Emilia Szállás (Eötvös University) and identified by Lengyel et al. (2005) at DSMZ, Braunschweig, Germany. The respective nematodes, except for *Steinernema feltiae* NY (supplied by A, Fodor) were obtained from Byron Adams, Brigham Young University.

Unrelated test organisms

Escherichia coli S17 λ^{pir} pKNOCK was obtained from Dr. Eric Martens and Dr. Heidi Goodrich-Blair, University of Wisconsin, Madison. Mastitis isolates; *Staphylococcus aureus* (Staph 1-6), *E. coli* (Ec471, Ec673, Ec707, Ec727, Ec884 and Ec902) and *Klebsiella pneumoniae* (Kp696) (hereafter referred to as *Kle. pneumoniae*), were obtained from the mastitis laboratory at the OSU-OARDC, Wooster, OH and were the same used by Furgani et al. (2008).

Antimicrobial activity assays

Strains of *X. budapestensis* DSM 16342^T, *X. szentirmaii* DSM 16338^T, *X. innexi* DSM 16336^T, *X. ehlersii* DSM 16337^T, *X. cabanillassii* RIO-HU, *X. nematophila* ATTC 61061, and *X. feltiae* NYH) were tested for their activities and sensitivities to each others' anti-bacterial compounds. For comparison, the antibiotic activity of each strain was simultaneously tested against non-related gram-negative (*E. coli* Ec727 and *Kle. pneumoniae*) and gram-positive (*S. aureus* Staph6) targets. Since four of the seven *Xenorhabdus* species above have recently been identified (Lengyel et al., 2005) and since their general antibacterial activities have not yet been systematically investigated, prior to the above-mentioned comparative studies, their activity against the gram-negative and gram-positive targets were also determined by using the overlay and cell-free bioassay methods described by Furgani et al. (2008). The antibiotic activity of *Xenorhabdus* type strains DSM 16342, DSM 16338, DSM 16336 and DSM 16337 were tested in overlay bioassays and liquid medium bioassays unless otherwise stated.

1. Overlay bioassay: The general antibacterial activities of the type strains of the four new *Xenorhabdus* species were tested against three mastitis isolates (animal pathogens), Staph6, Ec727 and *K. pneumoniae*. The diameters (given in mm) of the inhibition circles were measured after five days.
2. Liquid medium bioassay: Liquid cell-free cultures of the four *Xenorhabdus* type strains were used tested on a laboratory (S17λ^{pir}) and two mastitis isolates (Ec707 and Ec902) of *E. coli*; mastitis isolates of *K. pneumoniae* and the gram-positive *S. aureus* (Staph6) with the method previously described (Furgani et al., 2008) to determine the maximum inhibiting dilution (MID) (minimum lethal concentration of the compounds with antibiotic activity). Seven serial dilutions of the cell-free culture were used: 0, 10, 20, 30, 40, 50 and 60% by volume. Four replicates of the 140 combinations of four source strains, five target strains and seven concentrations were performed. The least concentrations (maximum dilution, MID) inhibiting the growth of the target bacteria were recorded.
3. Dilution assay: To confirm that the effect is generally bacteriotoxic as found by Böszörményi et al. (2009) against *Erwinia amylovora*, a serially diluted, stationary-phase, cell-free culture of *X. szentirmaii* was assayed in 12-well plates against *K. pneumoniae.* The MID% values were determined as follows: 25 µl aliquots were taken from plate wells where no growth was observed to quantify cell changes. Aliquots were serially diluted in micro-titer wells with PBS from 10^{-1} down to 10^{-6}. Two replicates of 50 µl each were plated on LBA containing 75 µg of carbenicillin (Kp696 is resistant to this antibiotics). After the plates were incubated at 37°C O/N, colonies were counted and colony-forming units (CFU) /ml were calculated. For controls, 44 µl of 10^{-1} O/N of Kp696 culture were placed in 2.2 ml of LB in the well of a 12-well plate, mixed, and 50 µl samples plated at 0 and 2.5 h after incubation at 37°C.
4. Cross-tolerance bioassay: To test anti-Xenorhabdus activity of the cell-free medium and sensitivity of the cells of other *Xenorhabdus* strains, cell-free cultures of seven *Xenorhabdus* species were tested against those same *Xenorhabdus* species in separate 8 x 12-well microtiter plates. Two replicates of each of 6 concentrations (0, 20, 30, 40, 50 and 60% in columns 1 - 6 and 7 - 12) were carried out in 250 µl, containing 240 µl of cell-free culture diluted with 2 x LB and 10 µl of a 10 x diluted overnight [$(O/N)^{-10}$] culture of the bacterial cells (Furgani et al., 2008). Rows A–H was inoculated with cells of *X. nematophila, X. cabanillassii* RIO; *X. bovienii* NYH, *X. budapestensis, X. szentirmaii X. ehlersii* and *X. innexi*. Staph6 was also included as a positive control. Plates were incubated at 25°C for 120 h and evaluated every 24 h. A similar experiment was conducted in which cells of *K. pneumoniae*, six *S. aureus* (Staph1, Staph2, Staph3, Staph4, Staph5 and Staph 6) and six *E. coli* (Ec471, Ec673, Ec707, Ec727, Ec884 and Ec902) mastitis isolates, plus a non-pathogenic *E. coli* (S17□^{pir}) strain were exposed to the same cell-free *Xenorhabdus* cultures with the same procedure.

Quantitative elimination of *Xenorhabdus* antimicrobial activities from cell-free media

The complete details for removing antibiotic activity from the cell/free liquid cultures were reported by Böszörményi et al. (2009) and are summarized below. Cells and media were first separated by centrifugation. Amberlite® XAD 1180 (Acros Organics, NJ, USA) adsorbed antibiotic active ingredients from cell-free cultures as follows: 10 g of the adsorbent were suspended in 100 ml of sterile distilled water, autoclaved at 121°C for 20 min, then treated with 1% sterile HCl for 1 h and with 1% sterile NaOH for another hour. After 3 washings and centrifugation with sterile distilled water, the pH was adjusted to 7.8 and the adsorbent kept overnight at 4°C. One ml of this adsorbent was added to 25 ml samples of 6-d cell-free *Xenorhabdus* cultures, and incubated for 24 h in Beckman centrifuge tubes in a shaker incubator (200 rpm) at 25°C. The adsorbent with the antibiotic compounds was removed by centrifugations at 3000 and 5000 G. The pellet was re-suspended in 25% methanol and stored at 4°C for further tests. To check for residual antibiotic activities, the Amberlite-extracted supernatants were serially diluted and tested in 8 x 12-well microtiter plates as described above.

Chemical and thermal stability of Xenorhabdus antibiotics

The chemical stability of the *X. budapestensis* antibiotics was evaluated by re-inoculation experiments. Three replicates of 40% cell-free cultures were inoculated in test tubes with *Kle. pneumoniae* incubated at 37°C for five days and the cell growth evaluated using a spectro-photometer (510 nm). The cultures were then re-inoculated with the same test organism. The growth was monitored and evaluated after another five days. The cultures were then re-inoculated for a third time and the cell densities (OD values) were again monitored after an additional five days.

The thermal stability of the *X. budapestensis* antibiotic complex was compared with the other *Xenorhabdus* species. Samples were autoclaved at 121°C for 10 min and tested against the gram-positive *S. aureus* Staph6 and gram-negative *K. pneumoniae* mastitis isolates, the other *Xenorhabdus* strains and the non-

pathogenic laboratory strain of *E. coli* λ^{pir} pKNOCK. Visual observation and plating techniques were used to monitor the growth of the test organisms.

Statistical analysis

For antimicrobial activity of *Xenorhabdus* strains on the gram-positive and gram-negative bacteria determined in overlay tests, the sizes (diameter in mm) of the inactivation zones were analyzed as a fully randomized (two-way) design with 'target' bacteria against the *Xenorhabdus* ('source') bacteria (see individual data in Table 1). Specific hypotheses were tested using some contrasts. The effectiveness of the type strain of *X. budapestensis* (DSM 16342) and that of *X. szentirmaii* (DSM 16338) on the same targets were compared. The interaction of the *X .budapestensis* and *X. szentirmaii* with the gram-positive and gram-negative targets was also tested. In addition, Fisher's protected LSD was used to test mean differences between the inhibition zones of the 'source' bacteria and between the 'target' bacteria. Results of the liquid medium bioassays of the four new *Xenorhabdus* species against *K. pneumoniae*, *S. aureus* and *E. coli* strains were analyzed as 6 x 8 factorial experiments. Hypotheses were tested by contrasts and Fisher's protected LSD test was used to compare means within each treatment axis. The mean and standard deviations of the maximum inhibiting dilutions were computed and the results are presented as a histogram with error bars (Figure 1). The dilution assay is presented as a regression of log [MFU/ml] against time with standard error bars (Figure 2). The cross-tolerance bioassays aiming at determining the maximum inhibiting dilutions of *Xenorhabdus* against each other were analyzed as a 6^2 factorial experiment. All analyses were performed using Statistica 6.1 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS

General antibacterial activities of Xenorhabdus species in two bioassays

Overlay bioassay

We tested the hypothesis that the four new *Xenorhabdus* species (represented by DSM 16342, DSM 16338, DSM 16336 and DSM 16337) produce effective antibiotics of different strength against gram-positive and gram-negative targets. Analysis of variance showed highly significant differences between the 'source' species' effects ($F_{5,108}$ = 1238, p = 0) and between the 'target' species susceptibility ($F_{2,108}$ = 420, p = 0). There was a strong interaction between "source species effect" and "target species susceptibility", ($F_{10,108}$ = 62.2, p < 0.0001). This indicates that that the sensitivity to and the efficacy of anti-*Xenorhabdus* compounds on *Xenorhabdus* spe-cies are not independent. The higher efficacy of *X. szentirmaii* on gram positive bacteria were reflected when the so-called "specific hypotheses" were tested. We con-cluded, that the type strains (*X. budapestensis*, DSM 16342 and *X. szentirmaii*, DSM 16338) significantly dif-fered in their effect ($F_{1,108}$ = 73.4, p < 0.0001) from each other. We found, that the non-related gram-negative and gram-positive bacteria differed in susceptibility ($F_{1,108}$ = 692, p = 0). The data also indicate, that the two gram-negative species, *Kle. pneumoniae* and *E. coli* differed from each other concerning susceptibility to *Xenorhabdus* antibiotics ($F_{1,108}$ = 147, p = 0). The very high *F*-ratios reflect the very small variation in response of the targets in the overlay test, resulting in very small residual va-riances. These apparently large statistical differences may not necessarily denote important biological effects. In addition a Fisher's protected LSD test was performed to examine the relative effects of the source strains and responses of the 'target' strains (individual data in Table 1). The range between the largest and smallest inhibition zones are certainly biologically meaningful showing, that *X. budapestensis* and *X. szentirmaii* should be consi-dered as strong, *X. innexi* as a medium and *X. ehlersii* as a very weak antibiotics producer. Each type strain exerted antagonistic effects on the gram-positive targets, but significant differences could be demonstrated between them. *X. ehlersii* exerted weak, *X. innexi* medium, *X. budapestensis* exerted a strong, and *X. szentirmaii* an even stronger activity against *Staphylococcus*. Of the six *S. aureus* strains Staph3 was significantly more tolerant to more *Xenorhabdus* anti-biotics than the others. (Only data of Staph3 and Staph 6 are given). The Gram-negative bacteria (*E. coli* and *K. pneumoniae*) were significantly more tolerant than the *S. aureus,* and *K. pneumoniae* was significantly more tole-rant than *E. coli*. The different *E. coli* strains reacted very similarly (only data of S17λ^{pir} and E.c. 727 are given). *Xenorhabdus ehlersii was* ineffective against Gram-negative targets. On the other hand, *X. budapestensis* and *X. szentirmaii* were both very effective. Antibacterial activity of *X. innexi* was medium on *E. coli*, but rather weak against *K. pneumoniae*. There were significant differences between the inactive *X. ehlersii*, the weak *X. innexi* and the other two, very active (*X. szentirmaii* and *X. budapestensis*) species concerning their effects on gram-negative targets.

Liquid medium bioassay

Figure 1 shows the MID values of the six-day-old cell-free cultures of strains of the four new species against one strain of *S. aureus* (Staph4), two virulent (Ec707 and Ec902) and one avirulent (S17λ^{pir}) strains of *E. coli* and the virulent #696 strain of *K. pneumoniae*. The type strains of *X. budapestensis* and *X. szentirmaii* were superior antibiotic producers in these tests as well. *X. innexi* was moderate and *X. ehlersii* again produced very weak antibiotics.

Dilution assay

Figure 2 demonstrated a bacteriotoxic rather than a bacteriostatic effect of cell-free cultures of *X. szentirmaii*.

Table 1. Antibacterial activities of four §novel *Xenorhabdus* species on three mastitis isolates in overlay bioassays in comparison with previously tested *Xenorhabdus*.

Source species and strain	Target species, strains		
	Diam. (mm) of the inactivation zone Mean ± S.E		
***Xenorhabdus* species, Strain**	***S. aureus***	***E. coli***	***K. pneumoniae***
	Staph 6	**E.c. 727**	**Isolate 696**
§*X. budapestensis*, DSM 16342^T	$^{a}60\ 0+/-\ 7.2_A$	$^{a}65.0+/-0.5_A$	$^{c}49.2\ +/-\ 1.8_B$
§*X. szentirmaii* , DSM 16338^T	$^{aa}73.7\ +/-\ 2.0_{AA}$	$^{a}64.1\ +/-\ 2.7_A$	$^{c}45.7\ +/-\ 3.5_B$
§*X. innexi* DSM 16336^T	$^{c}45.0\ +/-\ 3.5_B$	$^{c}44.8+/-2.1_B$	$^{d}39.8\ +/-2.1_B$
§*X. ehlersii* DSM 16337^T	$^{e}32.0\ +/-2.1_D$	$^{f}0_E$	$^{f}0_E$
X. nematophila ATCC 19061^T	$^{b}53{\cdot}3 \pm 4{\cdot}4_B$	$^{d}41{\cdot}0 \pm 0{\cdot}6$	$^{e}26{\cdot}0 \pm 1{\cdot}7_D$
X. nematophila DSM 3370^T	$^{b}54{\cdot}2 \pm 1{\cdot}3_B$	$^{c}49{\cdot}5 \pm 1{\cdot}1_B$	$^{d}34{\cdot}1 \pm 3{\cdot}2_C$
X. bovienii DSM4766^T	$^{e}33{\cdot}9 \pm 3{\cdot}8_C$	$^{e}29{\cdot}7 \pm 1{\cdot}2_D$	$^{e}27{\cdot}3 \pm 4{\cdot}4_D$
X. bovienii$_{NYH}$	$^{c}42{\cdot}5 \pm 4{\cdot}2_B$	$^{d}33{\cdot}0 \pm 1{\cdot}5_C$	$^{d}34{\cdot}3 \pm 1{\cdot}9_C$
X. bovienii$_{SF22}$	$^{d}37{\cdot}5 \pm 3{\cdot}1_C$	$^{f}0_E$	$^{f}0_E$
X. cabanillassi RIO-HU	$^{a}60{\cdot}7 \pm 0{\cdot}7_A$	$^{b}54{\cdot}8 \pm 3{\cdot}5_B$	$^{d}32{\cdot}4 \pm 3{\cdot}5_D$

Mean inhibition zone diameters followed by the same latter are not significantly different from each other by Fisher's protected LSD test. Data concerning the source species (lower case on the left of the data, as superscript) and those concerning the target species (upper case on the right of the data. as subscript) were analyzed separately. The data from *X. bovienii, X. nematophila* and *X. cabanillassii* are "controls" with previously published activity (Furgani et al., 2008). ^{T}Types strain deposited in DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brauschweig, Germany) or in ATCC (American Type Culture Collection, Rockville, MD, USA).

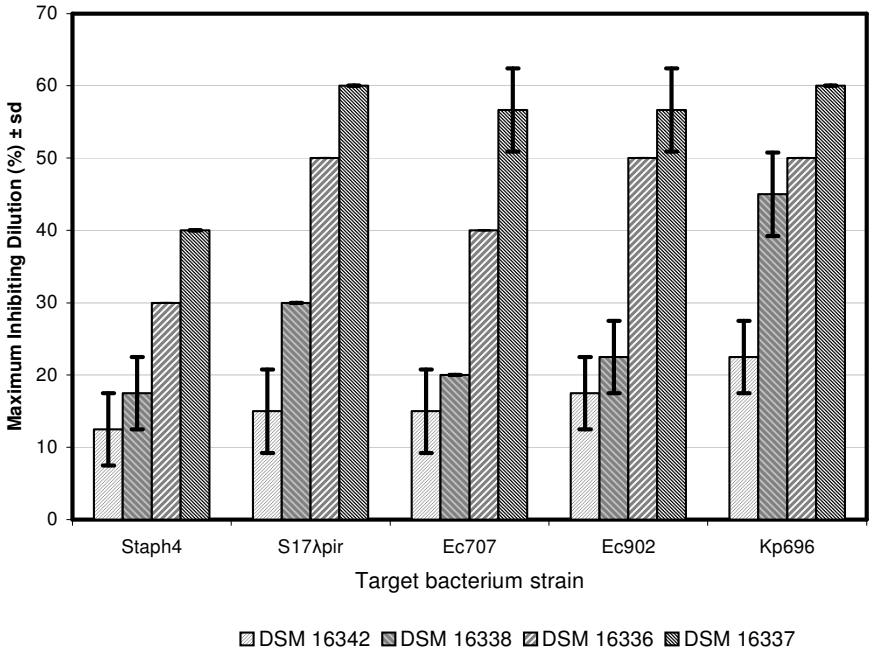

Figure 1. Effects of cell-free cultures of four novel *Xenorhabdus* species on non-related Gram-negative and Gram-positive bacteria in liquid culture bioassays. Mean concentrations of *X. budapestensis, X. szentirmaii*, *X. innexi* and *X. ehlersii* at their maximum dilutions having an inhibitory effect against *S. aureus* (Staph6), *E. coli* (S17λ^{pir}, Ec707 and Ec902) and *K. pneumoniae* (Kp696). The lower the dilution % the higher the concentration of the antimicrobial compounds of the media. Error bars are standard deviations.

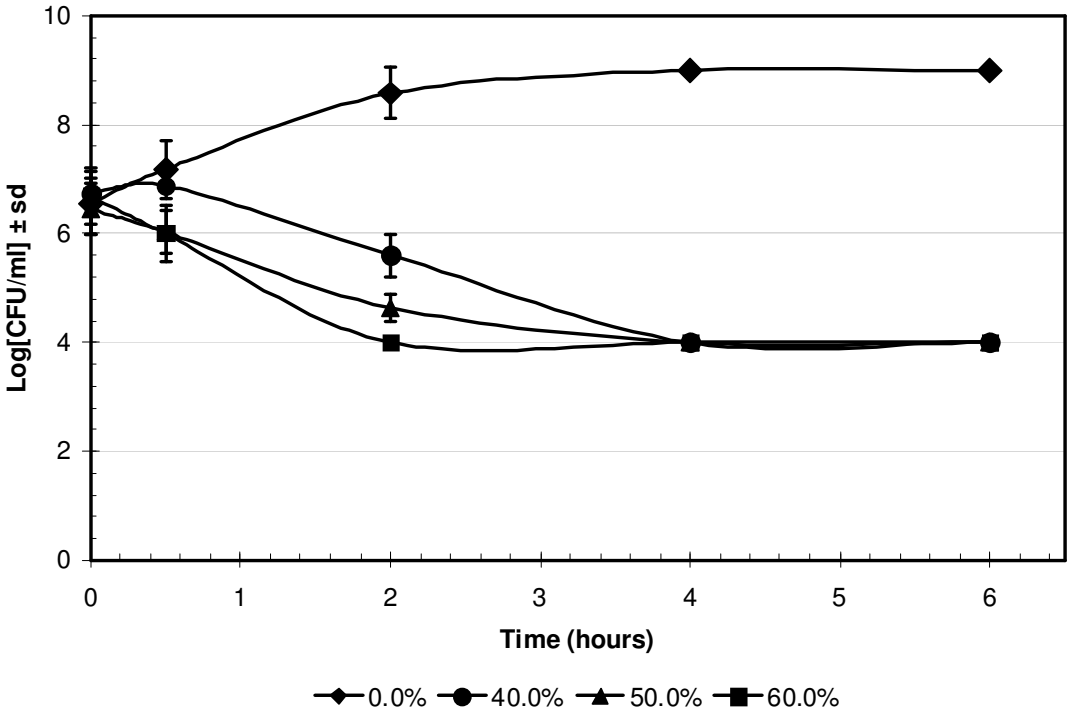

Figure 2. Bacteriotoxic effect of diluted cell-free cultures of *X. szentirmaii* on *K. pneumoniae* (Kp696). Error bars are standard deviations.

The original number (-10^6/ml) of *K. pneumoniae* cells in the control cultures increased three orders of magnitude (> 10^9/ml) within 4 h, while the number of cells in the treatments containing 40, 50 and 60% of the six-day old cell-free media of *X. szentirmaii* decreased significantly to < 10^4 within 4 h and did not rebound within 6 h. Similar results were obtained by Böszörményi et al. (2009) in assays against *E. amylovora* with both *X. szentirmaii* and *X. budapestensis* in different experimental conditions.

Cross-tolerance bioassay

The hypothesis that each of seven *Xenorhabdus* strains would exert antagonistic effects on each other and that these antibiotics are at least partly different from those active on non-related gram-negative bacterial isolates was tested and proven. We expected there to be species differences both in the production of these anti-Xenorhabdus compounds and in their sensitivities to them. We also tested whether anti-Xenorhabdus activity of the cell-free medium and sensitivity of the cells of other *Xenorhabdus* strains anti-Xenorhabdus activities were related. Analysis of variance showed highly significant differences between the “source species effects” ($F_{6,147}$ = 1438, p = 0) and between the “target species susceptibility” ($F_{20,147}$ = 867, p = 0). Their interaction was also highly significant ($F_{10,108}$ = 138, p = 0). Specific hypotheses were tested by contrasts: the type strains of *X. budapestensis* and *X. szentirmaii* differed in effect ($F_{1,108}$ = 1376, p = 0); as well as in susceptibility to the *Xenorhabdus* antibacterial substances ($F_{1,108}$ = 168, p = 0). The gram-positive and gram-negative bacteria differed in susceptibility ($F_{1,147}$ = 765, p = 0). Taken as a group, the *E. coli* bacteria differed in susceptibility to *X. budapestensis* and *X. szentirmaii* ($F_{1, 147}$ = 700, p = 0). *E. coli* and *K. pneumoniae* differed in susceptibility to *Xenorhabdus* anti-bacterial agents ($F_{1,147}$ = 514, p = 0). These findings indirectly demonstrate that the compounds active on non-related targets probably are not the same as those active on the related targets. The ‘within treatment’ variation was so small that the results were essentially deterministic (p = 0). A Fisher’s protected LSD test was performed to examine the relative effects of the ‘source’ strains and responses of the ’target’ strains (Tables 2a and b). The largest MID percent were approximately twice the minimum values indicating real biologically meaningful differences between the extremes.

The general antibacterial activities of the cell-free cultures on non-related bacteria do not correlate well with their anti-*Xenorhabdus* activities. Despite its strong antimicrobial activity on all mastitis bacteria, the anti-*Xenorhabdus* activity of *X. budapestensis* is moderate. On the other hand, the cell-free cultures of *X. ehlersii*, which were extremely weak against non-related bacteria, were surprisingly toxic for the cells of *Xenorhabdus*

Table 2a. Four novel *Xenorhabdus* species Effects of cell-free *Xenorhabdus* liquid cultures on related and non-related bacteria. LC95 as (V/V) %# expressed as MID%.

Source (Antibiotics-producing species)	*Xenorhabdus*			
	innexi	***ehlersii***	***szentirmaii***	***budapestensis***
Target species Strain	**DSM 6336^T**	**DSM 16337^T**	**DSM 63338^T**	**DSM 16342^T**
X. innexi DSM 16337^T	> 60	50	30	> 60
X. ehlersii DSM 16336^T	> 60	40	< 20	> 60
X. nematophila ATCC 19061^T	> 60	40	< 20	> 60
X. budapestensis DSM 16342^T	> 60	40	> 60	> 60
X. szentirmaii DSM 16338^T	> 60	40	> 60	> 60
X. cabanillassii RIO-HU	> 60	30	< 20	30
X. bovienii NYH	< 20	< 20	< 20	< 20
E. coli S17λ^{pir}	40	> 60	30	30
E. coli E.C. 727	40	> 60	30	30
K. pneumoniae # 696M	50	> 60	40	40
S. aureus Staph 6	30	40	30	30
S. aureus Staph 3	40	> 60	40	40

Table 2b. Three other *Xenorhabdus* species effects of cell-free *Xenorhabdus* liquid cultures on related and non-related bacteria. LC95 as (V/V) %# expressed as MID%.

Source (Antibiotics-producing species	*Xenorhabdus*		
	nematophila	***bovienii***	***cabanillassii***
Target species Strain	**ATTC19061**	**NYH**	**RIO-HU**
X. innexi DSM 16337^T	> 60	40	50
X. ehlersii DSM 16336^T	> 60	< 20	50
X. nematophila ATCC 19061^T	> 60	< 20	> 60
X. budapestensis DSM 16342^T	50	40	30
X. szentirmaii DSM 16338^T	50	30	< 20
X. cabanillassii RIO-HU	< 20	< 20	< 20
X. bovienii NYH	< 20	< 20	< 20
E. coli S17λ^{pir}	40	30	30
E. coli E.C. 727	40	40	< 20
K. pneumoniae # 696M	50	50	50
S. aureus Staph 6	30	30	30
S. aureus Staph 3	40	40	20

E. coli = Escherichia coli; *Kle = Klebsiella; S. aureus = Staphylococcus aureus.* E.c. 727 and # 696 are mastitis isolates. DSM = Type strain, deposited in DSMZ, (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brauschweig, Germany); ATCC^T= Type strain, deposited in ATTC (American Type Culture Collection, Rockville, MD, USA.) MID% = Maximum Inhibiting Dilution, (the minimal concentration of antibiotics, expressed in Volume/Volume ($V_{cell\text{-}free\ culture}$/ $V_{LB\ medium,}$) %). The lower the MID%, the larger are the concentration of the active compound(s) #n=12.

species, except for those of *X. budapestensis, X. szentirmaii* and its own. *X. bovienii* NYH showed the strongest and rather uniform anti-*Xenorhabdus* activity, but was most sensitive to the other anti - *Xenorhabdus*

activities. On the other hand, the anti-*Xenorhabdus* activity of *X. innexi* was the least potent (except for its effect on *X. ehlersii*), but was highly resistant to each of the other anti-*Xenorhabdus* compounds. This negative correlation was not observed in other species. *X. ehlersii*, which was very weak against non-related bacteria, was surprisingly effective against *X. nematophila* and *X. cabanallasii,* and completely inhibited *X. bovienii* at < 20%. This clearly demonstrates that the general anti-bacterial compounds of *Xenorhabdus* species are rather different from those with anti-Xenorhabdus activity. Some overlap cannot be excluded.

X. szentirmaii produced both efficient anti-Xenorhabdus compounds and large spectrum antibiotics. Its antibiotics were effective against *K. pneumoniae* and *E. coli.* Its anti-anti-Xenorhabdus compounds were effective against all the other *Xenorhabdus* species except for *X. budapestensis*. The cell-free culture of *X. cabanallasii* was toxic to all the other *Xenorhabdus* species, except for X*. nematophila.* The cell-free cultures of *X. nematophila* proved completely ineffective on the cells of *X. innexi* and *X. ehlersii* (as well as on its own cells). It was slightly ineffective on *X. budapestensis* and *X. szentirmaii* (at ≥ 40%) but completely inhibited the cell propagation of *X. cabanillassii* and *X. bovienii* at < 20% dilution.

Elimination of *Xenorhabdus* antimicrobial activity by Amberlite^{R} XAD 1180

Amberlite^{R} XAD 1180 polymeric adsorbent adsorbed all antibiotics against *Xenorhabdus* strains as well as against mastitis isolates. No trace activity was detected in any Amberlite-treated *Xenorhabdus* cell-free culture. The activities could also quantitatively be eluted and retested. Amberlite adsorbs compounds with specific polarity; however, it does not allow us to make any conclusions concerning the chemical nature of the biologically active compounds. However, from practical point of view, it could be useful for providing an option for selective elution of compounds with different biological activity in the future.

Chemical and thermal stability of *Xenorhabdus* antibiotics

The antibacterial activity of the cell-free cultures of *X. budapestensis* and *X. szentirmaii* did not decrease after being stored at room temperature for 6 days, indicating the compounds are chemically stabile. No growth of *K. pneumoniae* cells was detected at 5, 10 and 14 days(OD= 0.03, 0.02 and 0.02, respectively) in a 40% dilution of *X. budapestensis* cell-free media, indicating both the cytotoxic nature and the stability of the active compound(s), at 37°C. On the other hand, in the re-inoculated 40% *X. szentirmaii* cultures, no growth of the *K. pneumoniae* cells was detected in the first 4 days (OD = 0.04), but they started to growth later on (OD >1 at the 5, 10 and 14th), indicating the antibiotics were cytotoxic, but they decayed sometime after 4 - 5 days at 37°C.

Autoclaving at 121°C for 10 min did not influence the antibiotic activities of the cell-free cultures of *X. budapestensis* (Table 3). Both non-autoclaved and autoclaved samples stored at 15°C for 3 years retained antimicrobial activity against three different *Leishmania* species (B. S. McGwire*,* personal communication).In comparison, a similar autoclave treatment reduced, but did not completely eliminate, the antibiotic activities of some *X. nematophila* strains. The cell-free cultures of the autoclaved *X. nematophila* N2-4 and AN6/I strains lost some of their activities. The antimicrobials of *X. cabanillassi* and *X. szentirmaii* were heat stabile like those of *X. budapestensis*. These data indirectly support the hypothesis that the antibiotics from different species and strains are chemically different.

DISCUSSION

We compared the antimicrobial activities of the type strains of four recently discovered species, (*X. ehlersii*, *X. innexi, X. szentirmaii* and *X. budapestensis*, Lengyel et al., 2005) by adopting two previously published (Furgani et al., 2008) bioassays on non-related gram-negative, gram-positive bacteria and on each other. In the intra-generic studies we involved representative strains of three other previously studied *Xenorhabdus* species: *X. nematophila* (Völgyi et al., 1998, 2000); *X. bovienii*, NY and *X. cabanillassii* (Furgani et al., 2008). We hypothesized that the different species will act differently against different targets. Researchers frequently test EPB antibiotics only on one target, usually on a sensitive one like *Micrococcus luteus* (Park et al., 2009). We found, that in general, the Gram-positive bacteria, such as *S. aureus* are more sensitive to every *Xenorhabdus* antibiotic than the gram-negative bacteria, such as *E. coli and K. pneumoniae*, as reported by Furgani et al. (2008). *K. pneumoniae* was significantly and consequently more tolerant than *E. coli to Xenorhabdus* antimicrobials in every bioassays Also, there were significant differences between the four newly isolated *Xenorhabdus* species with regard to their antibiotics production and/or activities. *X. ehlersii* was barely active against gram–positive, and completely ineffective against non-related gram-negative targets. *X. innexi* was similarly active against both gram positive and non-related gram-negative targets, indicating that its antibiotic profile was different from that of *X. ehlersii,* as well as from those of X*. szentirmaii* and *X. budapestensis*. These latter two were by far the best antibiotic producers of all the *Xenorhabdus* species studied so far (Lengyel et al., 2005; Furgani et al., 2008; Böszörményi et al., 2009). Their antibiotic activities were

Table 3. Thermal stability of *Xenorhabdus* antibiotics bacteriotoxic activities (maximum inhibiting dilution) before and after autoclaving on *K. pneumoniae* Kp696.

***Xenorhabdus* species, strain**	**Overlay bioassay**	**Direct bioassays of six day cell-free cultures**					
	Inhibition zone	**Before autoclaving V/V% in 2xLB**			**After autoclaving V/V% in 2xLB**		
	(mm)	**40**	**50**	**60**	**40**	**50**	**60**
X. szentirmaii, DSM 16338	51.0 ± 1.73	I	I	I	[b]+++	I	I
X. budapestensis, DSM 16342	39.0 ± 1.73	[a]I	I	I	I	I	I
X. cabanillassii, RIO-HU	36.0 ± 1.15	I	I	I	I	I	I
X. nematophila, DSM 3370	34.3 ± 0.88	I	I	I	+++	I	I
ATTC 19061	34.3 ± 0.88	I	I	I	+++	I	I
AN6/I	34.3 ± 1.45	I	I	I	+++	+++	I
N2 - 4	28.0 ± 0.58	+++	I	I	+++	+++	I

[a]I = complete inhibition, no cell propagation, [b]+++ = no inhibiting effects, the test organism perfectly grows, that is significant differences between the antimicrobial activities of autoclaved and non-autoclaved cell-free cultures. (N2 - 4 was not effective on 40% v/v dose either before or after being autoclaved on *K. pneumoniae*).

comparable to those of *X. nematophila* strains used in this and previous studies, to *X. cabanillassii* and to the best strains against non-related gram-negative and gram-positive targets. But this does not indicate similar antibiotic profiles of the latter four, since with Gram-positives, *X. szentirmaii* was by far the best, while with non-related gram-negatives, *X. budapestensis* was the best. This was especially unambiguous in tests on *K. pneumoniae.*

We could not demonstrate any correlation between the anti-*Xenorhabdus* and general antibacterial activities. For instance, the moderate antibiotic producer, *X. innexi*, *was* most resistant to the antibiotics of the other *Xenorhabdus* strains. On the other hand, *X. bovienii* NY produced the most potent anti-Xenorhabdus antibiotics. It was a good, but not the best, antibiotic producer when tested on non-related gram-negatives, but was the most sensitive to the antibiotics of the other species. The best anti-mastitis antibiotic producers, *X. budapestensis* and *X. szentirmaii*, were rather vulnerable to the antibiotics of other species. Antibiotic activity and compounds of the *Xenorhabdus* symbiont of *S. riobrave* (later identified as *X. cabanillassii* by Tailliez et al (2006)) was discovered by Isaacson and Webster (2002). They did not test them on other *Xenorhabdus* species. *X. cabanillassii* had the second best, after *X. bovienii* NY, activity on other *Xenorhabdus* species. *X. budapestensis* was the best antibiotics producer against non-related gram-negatives, but was antibiotically active only against *X. cabanillassii* and *X. bovienii* NYH. Since *X. szentirmaii* did not inactivate *X. budapestensis*, we may simultaneously apply the two strongest antibiotics producers.

The strikingly high level of interspecies diversity of small-molecule antibiotics used to be considered as inhibiting a range of bacteria and fungi, (Webster et al., 2002). Many of them are of pharmaceutical and/or agricultural importance, including *Staphylococcus* and coliform species (Furgani et al., 2008), and *E. amylovora* (Böszörményi et al., 2009). In contrast, macro-biomolecules such as bacteriocins (xenorhabdicins) used to be considered as inhibiting the growth of closely related *Xenorhabdus* species (Boemare et al., 1992; Thaler et al., 1995, 1997; Webster et al., 2002). Banerjee and his associates described a two-gene cluster encoding a bacteriocin, xenocin, and the cognate immunity protein in *X. nematophila*. The two genes, *xcin*A and *xim*B, are present in the genome as a single transcriptional unit, which is regulated under SOS conditions. Xenocin also inhibited the growth of two *Xenorhabdus* isolates. It was demonstrated that $^{+++}$Fe depletion acts as a common cue for synthesis of xenocin by *X. nematophila* and sensitization of the target strains to the bacteriocin (Singh and Banerjee, 2008). These findings may explain why the total antibacterial activities, including both general and anti-*Xenorhabdus* compounds could be completely removed with the same adsorbent from cell-free *Xenorhabdus* media. New data from the Forst Laboratory (Park et al., 2009) indicates that the picture maybe not so simple. A 14 gene (*xcnA-N*) cluster involved in xenocoumacin 1 (Xcn1) and xenocoumacin 2 (Xcn 2) production was identified by insertional inactivation of non-ribosomal peptide synthetase (*xcnA* and *xcnK*) and polyketide synthetase (*xcnF xcnH xcnL*) genes. Residual antibiotic activity remained detectable in the mutant strains due to non-xenocoumacin antibiotics. The question is whether *X. budapestensis*, *X. szentirmaii* and *X. innexi* also have xenocoumacins as the main antibiotics. The comparison of the antibiotic activities of representative strains of different *Xenorhabdus* species on different targets, including closely related, non-related gram-negative and gram-positive bacteria, as well as *Leishmania* species (B. S. McGwire, personal communication), suggest that different compounds might be active on different 'target' taxa. For instance, *X. ehlersii*,

which is completely inactive against non-related gram negatives and only slightly active against gram-positives, was quite active against related species. However, *X. innexi*, which seems equally active against non-related Gram-negative and Gram-positive bacteria, was almost completely inactive against other *Xenorhabdus* species. Unexpectedly, antimicrobial compounds of *X. innexi were* more active against *Leishmania donovani* than those of *X. budapestensis* or *X. szentirmaii* (McGwire et al., in preparation). Both *X. budapestensis* and *X. szentirmaii*, the most active ones against non-related gram-negatives and gram-positives, exert a rather moderate effect on other *Xenorhabdus* species. Re-inoculation experiments showed the active compounds of *X. budapestensis* were stabile at 37°C for up at least for two weeks. Under the same conditions, the activity of *X. szentirmaii* was lost within five days in room temperature. The partially purified fractions isolated from *X. budapestensis* were stabile at least for three months in a refrigerator if the pH is -5.6, but not at lower pH (Szentirmai, personal communication). These data indirectly show that the antibiotic profiles of different *Xenorhabdus* species are different. As demonstrated in the dilution experiment, the antibacterial activities are cytotoxic rather than cytostatic. From both a theoretical and applied view, it is important that the autoclaved cell-free cultures did not loose their antibiotic activity. The anti-Xenorhabdus activities of the cell-free media and sensitivities of the cells to anti-Xenorhabdus activities of other *Xenorhabdus* strains were negatively correlated in two of seven instances (*X. bovienii, X. innexi*).

The biological significance of this is not clear, but in light of the results of Singh and Banerjee (2008) we cannot exclude the possibility that there are some cues other than $^{+++}$Fe depletion which might stimulate synthesis (or activation) and at the same time increase the sensitization to the bacteriocin of the producing strain. Clearly, these results provide a model system for looking at resistance mechanisms in bacteria. As for applied aspects, fortunately the cells of the extremely potent *X. budapestensis* were completely tolerant to anti-Xenorhabdus compounds produced by *X. szentirmaii*. Also, the cells of *X. nematophila* and *X. budapestensis* proved tolerant to the antibacterial compounds of each other. This provides a possibility of using them simultaneously to multiply their efficacy for suppressing animal or plant microbial pathogens.

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