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Antimicrobial Peptides for Combating Eukaryotic Pathogens: Anti-Histomonal, and Anti-Leishmanial Potential of Antimicrobial Peptides Released by Entomopathogenic Nematode Symbiont Bacteria

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Abstract Antimicrobial peptides provide a powerful toolkit for combating multidrug resistance. Combatting eukaryotic pathogens is complicated   by the fact, the intracellular drug targets in the eukaryotic pathogen are frequently homologs of cellular structures of vital importance in the host organism. The entomopathogenic bacteria, (symbionts of entomopathogenic nematode species), release a series of non-ribosomal templated antimicrobial peptides, some may be potential drug candidates. The ability of an entomopathogenic nematode /entomopathogenic bacterium symbiotic complex to survive in a given polyxenic milieu is a coevolutionary product, explaining that those gene complexes which are responsible for the biosynthesis of different non-ribosomal templated antimicrobial protective peptides (including those which are potently capable to inactivate the protist mammalian -pathogen *Leishmani*a and the gallinaceous bird pathogen *Histomonas*) are co-regulated. Our approach is based on comparative antimicrobial bioassays of the culture media of the wild-type and regulatory mutant strains. We concluded that *Xenorhabdus budapestensis* and *X. szentirmaii* are excellent sources of non-ribosomal templated antimicrobial peptides, that are efficient antagonists of the mentioned pathogens. Data on selective cytotoxicity of different cell-free culture media encourages us to forecast that the recently discovered "easy-PACId" research strategy is suitable for constructing EPB strains producing and releasing single, harmless, non-ribosomal templated antimicrobial peptides with considerable drug, (probiotic) -candidate potential.

1. Introduction

Multidrug resistance (MDR) is a great, and rapidly increasing challenge for scientists, in latest 8 decades. The different forms of MDR appearing in pathogen organisms of clinical and veterinary, (as well as of agricultural) importance partly due to the consequence of the astonishingly unprofessional applications in inaccurate doses providing optimal selective conditions and selective advantages for MDR organisms to original (wild-type) ones of the same species, [1] (Fodor et al., 2020). One attempt to overcome resistance problems is to test the general and specific antimicrobial potential to apply antimicrobial peptides (AMPs) [2] (Ötvös and Wade, 2014). AMPs are of great potential to combat MDR prokaryotes because of several reasons. First, there is an option of taking advantage from the option of using the remarkable toolkit called quantitative structure-activity relation (QSAR) [3] (Karelson, Lobanov, Katriczki) 1996); [4] (Gini, 2022) technology in designing novel antimicrobial-active sister molecules of a newly discovered natural drug-candidate AMP molecule. The drug designer has a powerful way to produce efficient, or even-more powerful (but physiologically less harmful analogs of AMPs which may overcome resistance problems in such a way. Furthermore, according to recent data, antibiotic-resistant bacteria tend to perform collateral sensitivity to AMPs, [5] (Lázár et al., 2018), and the mobility patterns of traditional AMP-resistance genes are different from that of the antibiotics-resistance genes, [6] (Kintses et al., 2019), [1] (Fodor et al., 2020).

We are interested in testing the antipathogenic potential of AMPs in controlling MDR eukaryotic pathogens. As for the two selected targets: they are *Leishmania. donovani* [7] (Chaoulitch, 1954); a mammalian pathogen, is transmittable by insects like cave-dwelling sand flies [8] (Dutra-Rêgo et al., 2022), and *Histomonas. meleagridis* is the causative factor (or etiological agent) of the life-threatening histomoniasis (or blackhead) disease) of birds (turkeys, chicken pheasants, first described in 1893 [9] (McDougald, 1993), [10] (McDougald, 2005), and have been studied in detail in the laboratory of [11] (Hess et al., 2015).

When planning experiments aiming at applying AMPs against eukaryotic pathogens, we have to take into consideration that the strategy of designing antimicrobials to control eukaryotic parasites (pathogens) must be different from that of designing antimicrobials to control prokaryotic pathogens, because targettable intracellular structures of a eukaryotic parasite (pathogen) biochemically might be similar to some intracellular structure of vital function in the eukaryote host to be protected.

We aim to learn whether any of those biosynthetic NR_AMPs (like fabclavine, [12], (Fuchs et al., 2012), [13] (Fuchs et al., 2014), phenazine, ˙[14] (Shi et al., 2019, or something else), produced by those entomopathogenic-nematode symbiont bacteria (EPB) we have been working on [15] Lengyel et al., et al., 2005), [16] (Fodor et al., 2022), were suitable for applying anti-protist drug candidates either against the intracellular parasite *Leishmania*, or the extracellular parasite *Histononas*. The arguments for choosing *in vitro* EPB liquid cultures as appropriate sources of efficient AMPs for this purpose are as follows: Obligate bacterial symbionts of entomopathogenic nematode species synthesize and release several non-ribosomal hybrid peptides [17] (Akhurst, 1982), of large target specificities which mainly serve to provide well-balanced pathobiome conditions for this symbiosis in polyxenic soil,- and cadaver environments [18] (Ogier, et al., 2020). These peptides are also considered potential sources of potent natural anti-microbial compounds, [17] (Akhurst, 1982). The motivation for choosing *in vitro* EPB liquid cultures as a source of efficient AMPs for this purpose are as follows: proven active on Gram-positive [19 (Furgani), [20] (Fodor et al., 2018), Gram-negative [22] (Böszörményi, et al., 2009) pathogenic bacteria, and oomycetes and different other plant pathogens, [23], (Fodor et al., 2010), [24] (Vozik et a., 2015) in our previous studies. We have reasons to believe that some of the autoclaved *Xenohabdus* cultures may not be harmful or toxic for toxic when added as a food supplement while some antimicrobial ingredients are still active, [21] (Fodor et al., 2023), we may not give up the option of considering potential probiotic applications, at least at experimental level.

As for the eukaryotic targets used in this study, the genus *Leishmani*a belongs to the Order Trypanosomatida, the Class Kinetoplastea, and the Phylum: Euglenozoa, [25] (Fernández-Arévalo et al., 2020), and is considered a blood parasite. It is the causative agent of leishmaniasis in humans, [26] (McGwire and Satoskar, 2014), and other mammals like dogs, [27] (Alvar et al., 2004); [28] (Dantas-Torres, et al., 2019].

The *Leishmania* pathogens act as intracellular parasites [29] (Walker et al., 2014). They retained the ability to produce extracellular vesicles. Both human,- and canine (caused by *Leishmania infantum*) can appear in a severe form called visceral leishmaniasis (VL), or kala-azar, [30] (Burza et al., 2018), [31] (Safavi et al, 2021). It has become to-endemic diseases have become global due to human migration, climate change, and anthropogenic disturbance, causing significant worldwide health and economic burden [32] (Altamura et al., 2022). As for the existing defense strategies: despite promising research activities, no clinically usable vaccine is available. In the absence of an effective vaccine, [33] (Mauël, 2002), the control of leishmaniasis relies exclusively on chemotherapy, [34] (Ibarra-Meneses et al., 2022). Each used clinical chemotherapy is based on very few chemotherapeutic agents, mainly antimonial derivatives (Sb). [35] Douanne et al, 2020]. As for available chemotherapies: Amphotericin B (AmB), a polyene macrolide antibiotic derived from actinomycetes, [36] (Golenser and Domb, 2006) has routinely been used in cases of visceral and canine leishmaniasis It had been selected from 200 known polyene agents, because of its moderate toxicity. It has been permitted for intravenous administration, [36] (Golenser and Domb, 2006). AmB acts in a membrane-destroying manner, [37] (Ellis, 2004), and has not provoked clinical resistance up to 1956, [36] (Golenser, and Domb, 2006). Since then severe drug resistance to both AmB and the other routinely used drug, miltefosine, (especially in *L. infantum* strains), appeared and continues spreading at alarming rates [34] (Ibarra-Meneses et al., 2022). *Leishmania* has always become an exceptional t model for studying microbial virulence, [38 [Chang, 2003). To outline the fascinating resistance mechanisms developed by *Leishmania* would require a long detailed review, so we want to refer to some publications instead ([35] Douanne et al, 2020], [20] (Golenser and Domb, 2006; [34] (Ibarra-Meneses et al., 2022). [39] (Segovia, 1994),

The other chosen target, *H. meleagridis* belongs to the genus *Histomonas*, a member of the family Monocercomonadidae within the class Trichomonada, in the class Trichomonadida of Phylum Metamonda.   Infections in turkeys may cause nearly 100% mortality. Outbreaks in chickens are more often marked by morbidity and subsequent recovery. The outbreaks in broiler breeder pullets at that time caused excessive losses from mortality (5 to 20%) culling, and overall poor flock performance. *Histomonas* parasites are carried by the eggs of the cecal worm *Heterakis gallinarum* enabling them to survive for long periods in the soil as a source of infection, [9] (McDougald, 1998), [10]] (McDougald, 1998), [11] (Hess et al., 2015). In the U.S. there were never any drug products available for the treatment of blackhead disease, [9] (McDougald, 1998), [10]] (McDougald, 1998), [11] (Hess et al., 2015). As for molecular taxonomy, and identification, the 5.8S, ITS-1, and ITS-2 rRNA regions were first sequenced [40] (Lollis et al. 2011). The most preferable remarkable reference gene to investigate gene expression as a symptom of the infection in selected organs from turkeys and chickens (called 60S ribosomal protein L13) has been identified [41] (Mitra et al., 2016), and used different infection models related to immunization, and vaccination, [42-43] (Kidane et al, 2016; (Mitra et al., 2018). The complete, annotated Histomonas reference genome sequence has been available, [44] (Palmieri et al., 2021). Histomoniasis is a fastidious disease in turkeys, with pathological lesions in the caeca and liver, sometimes with high mortality. In chickens, the disease is less fatal, and lesions are often confined to the caeca. In Europe, the disease was well controlled by applying nitroimidazoles and nitrofurans for therapy or prophylaxis, [11] (Hess et al., 2015). It infects a wide range of birds including chickens, turkeys, peafowl, quail, and pheasants, causing infectious enterohepatic lesions. Since their introduction into the market in the middle of the previous century, further research nearly ceased as outbreaks of histomoniasis occurred only very rarely.

With the ban of these drugs in the last two decades in North America, the European Union, and elsewhere, in combination with the changes in animal husbandry, the disease re-emerged, [11] (Hess et al., 2015). But at that time the previously applied drugs were banned. Consequently, research programs were set up in various places focusing on different features of the parasite and the disease.

For the first time studies were performed to elucidate the molecular repertoire of the parasite. In addition, research has been started to investigate the parasite's interaction with its host, [11] (Hess et al., 2015). New diagnostic methods and tools were developed and tested with samples obtained from field outbreaks or experimental infections. Some of these studies aimed to clarify the introduction of the protozoan parasite into a flock and the transmission between birds, [45] (Hauck et al., 2010). Finally, a strong focus was placed on research concentrated on the development of new treatments and prophylactic strategies, urgently needed to combat the disease, [11] (Hess et al., 2015).

As for vaccination, studies on the immune response against histomoniasis focus mainly on different traits of the adaptive immune system. Activation of toll-like receptors (TLR) leads to the interplay between cells of innate and adaptive immunity with consequences on B and T cell clonal expansion. Efforts for successful vaccinations are on the way for years, [46] (Lagler et al., 2021), but various challenges need to be addressed until the first ever developed vaccine based upon live flagellates in human or bird medicine can be marketed. Summarizing: the poultry industry works without approved prophylactics, therapeutics, or vaccines to combat histomonosis, [47] (Beer et al., 2022).

Meanwhile, numerous chemical compounds were also tested for their efficacy against *H. meleagridis* with varying outcomes. One of the explanations for these half-failures / half-successes may be the absence of reliable and reproducible *in vitro Histomonas* culturing techniques. *Escherichia coli* strongly supports the growth *of H. meleagridis* in a monoxenic culture, without influencing its pathogenicity, [48] (Ganas et al., 2012). Based on clonal cultures of *H. meleagridis*, monoxenic cultures have, to our knowledge for the first time, been established in a liquid medium. The fecal flora was exchanged for defined bacterial strains by selective destruction of the initial bacteria with a variety of antibiotics, keeping the flagellate alive. The growth of the protozoan parasite was found to depend on the bacteria, especially on their energy metabolism. *E. coli* was found to strongly support the growth of the parasite, whereas *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* were less efficient. Confocal laser microscopy showed that *H. meleagridis* could take up green fluorescent protein-tagged *E. coli* DH5α, suggesting that bacteria served as a food supply for the protozoa. By exchanging the bacterial flora for *E. coli* strain DH5α in *H. meleagridis* cultures that underwent continuous *in vitro* passages, it was possible to show that the *in vivo* attenuation process was independent of the bacteria. Furthermore, the gut flora in infected turkeys had no negative effect on the protozoan's virulence. Consequently, attenuation depends not on the bacteria in the culture but on the *in vitro* passages. Finally, the experiments provided evidence that the infection of turkeys with *H. meleagridis* enabled infection of the liver with *E. coli*, [48] (Ganas et al., 2012)..

In the following study identical monoxenic settings for cultures of the same *H. meleagridis* clonal strain in its virulent low passage and attenuated high passage form enabled a comparative analysis of parasite characteristics. For the first time, it could be shown that long-term *in vitro* cultivation led to a severe shift in cell morphology, with the occurrence of a very distinct phenotype expressing a flagellated and highly amoebic cell morphology. Furthermore, the attenuated parasites showed better growth rates and a higher tenacity when confronted with adverse conditions. During these experiments up to 100% of the parasites, both virulent and attenuated, assumed a completely rounded morphology elucidated by electron microscopy. The findings indicated that such previously reported cyst-like stages were a defense strategy of *H. meleagridis*, independent of the passage level *in vitro* and pathogenicity *in vivo*. In conclusion, long-term *in vitro* passaging of *H. meleagridis* led not only to an attenuation of the parasite, as previously demonstrated but also to a shift in the parasite's phenotype regarding morphology, growth behavior, and a higher level of tenacity, [49] (Gruber et al., 2017).

The option of the monogenic culturing technique opens the door to, preliminary attempts to protect birds with cultured attenuated histomonads forecasting the possibility of vaccination, [50] (Liebhart et al., 2017). Whether the special feature of the parasite's intricate interplay with bacteria *in vitro* and *in vivo*, can be considered mutualistic or a predator-prey one is a philosophical question, [51] (Bilic, and Hess, 2020). As for drug resistance, the first documented example of drug-resistant *H. meleagridis* appeared in the literature, and the antimicrobial potential of alkaloids (harmaline and harmalol) in crude extracts of the medical plant *Peganum harmala*, was tested against it, ˙[52] (Arshad et al., 2008).

After banning all previously used prophylactic and therapeutic drugs against *H. meleagridis* in the European Union, some benzimidazoles were only antiparasitic drugs, licensed for use in food-producing animals. [33] (Hauck and, Hafez, 2009). Benzimidazoles act on beta-tubulin, and the beta-tubulin sequence allows predictions about the efficacy of benzimidazoles. When sequenced and analyzed a part of the beta-tubulin gene of five *H. meleagridis* strains was found. all histomonal amino acid sequences predicted susceptibility to benzimidazoles. However, when the efficacy of five benzimidazoles, (albendazole, fenbendazole, flubendazole, mebendazole, and nocodazole), were tested on the 5 *H. meleagridis* strains *in vitro*, all tested drugs showed no efficacy, [38] (Hauck and, Hafez, 2009). Reduced sensitivity of *H. meleagridis* to nitarsone *in vitro* and *in vivo*. [54] [Abraham et al., 2014). Parasites are highly prevalent in poultry; thus, the management of parasites is a key component in the profitable production of poultry. The most common nematode parasite of poultry, *Heterakis gallinarum*, typically causes no direct pathology but is the vector of *Histomonas meleagridis*, a highly pathogenic protozoan parasite that causes blackhead disease. There are no approved treatments for *H. meleagridis*, making control reliant on controlling the helminth vector, [55] (Collins et al., 2022).

Herein we compared the spectrum of *in vitro* antimicrobial activity of cell-free conditioned culture media (CFCM) from three EPB species together with testing against *Leishmania donovanii, Trypanosoma cruzi*, *Histomonas meleagridis,* and as well as against panel of clinical bacterial and fungal isolates, as positive controls. Considering the roles of the bacterial symbiont in the EPN/EPB symbiosis, we suppose that ani-protozoal compounds must be coexisting with other antimicrobial compounds in the CFCM of EPBs. Therefore for reliable bioassays, we should use <u>appropriate controls</u>. Two main aspects ought to be taken into consideration: **(A)** To be able to distinguish between the specified antiprotist activity and general cytotoxicity of the future drug-candidate antimicrobial compounds in the CFCM. **(B)** To be able to identify the special anti-protist compounds. In this paper, we described the very first steps toward d these goals

<u>As for (**A**)</u>, one has to take into consideration that the lack of uniformity regarding the choice of cell types for cytotoxicity assays may lead to incomparable and inconclusive data. *In vitro* assays relying solely on non-phagocytic cell models may not represent a realistic result as the effect of an antileishmanial agent should ideally be presented based on its cytotoxicity profile against reticuloendothelial system cells. [56] (Brioschi et al., 2022). In the *Leishmania* studies, we used macrophage cell line J774A.1 **[57]** (Tominaga et al., 1998), [58] (Andreu et al., 2017), [59]. (Geroldinger et al., 2019), as cytopathogenecity control test organism, (see Materials and Methods).

In the *Histomonas* studies, we used permanent chicken liver cells (LMH (leghorn male hepatoma, LMH) cell line **[60]** (Kawaguchi et al., 1987), [61] (Amin et al., 2012), [21] (Fodor et al., 2023), as cytopathogenecity control test-organism, (see Materials and Methods).

As for (**B**), one has to take into consideration the following points: each NR_AMP-producing EPB cell is in the so-called primary (1^0) phase, or Phase 1, [62] (Forst and Nealson, 1996). In other terms, the phenotypic precondition for the antibiotic production (as well as for the capability of symbiosis) of the EPB species is the primary phase, [63] (Akhurst, 1980), [64] (Akhurst, 1982), [65](Boemare and Akhurst., 1994). From the aspect of NR_AMP production, it means, that in a liquid culture of the primary (1^0) cells (of each of the studied EPB species, strain, isolate) more than one biosynthetic AMPs are present at the same time. Each expresses antimicrobial (maybe antiprotist) activity, so the antimicrobial (antiprotist) activity of a CFCM sample represents a cumulative activity. But in an optimal test system, one would prefer to determine the antimicrobial activity of each of the single antimicrobial compounds one by one.

Since the discovery that the activity (that is, the „switch-on" / „switch-off" states) of those group genes (operons, biosynthetic gene complexes, BGCs) which are responsible for the primary-secondary phenotypic phase shift in of each known EPB strain, not only work under the control of the versatile regulator hexA, [66] (Joyce and Clarke, 2003), [67] (Langer et al., 2017) but also are coordinately regulated at a higher level (via *hfq*-gene controlled sRNS/ HexA-mRNA base pairing, more precizely, the Hfq-dependent sRNA, ArcZ, directly base-pairs with the HexA-encoding mRNA, [68] (Tobias et al., 2016), [69]′(Tobias et al., 2017), [70] (Neubacher, 2020)) it is possible to construct double-mutant strains from each EPB species (or isolate) following the genuine strategy called easyPACId (easy Promoter Activated Compound Identification) approach, [71] (Bode et al.,

2019), each of which biosynthesizes and releases only one single NR_AMP molecule into the culture media.

This discovery may be a starting point of the renaissance of *Xenorabdus* antimicrobial peptide research, [72] (Wenski et al., 2019), [73] (Wenski et al., 2020), [74] (Cimen et al., [75] 2022), (Gulsen et al., 2022).

 We also managed to reconstruct the "easyPACId" *hfq* deletion mutant strains from EMA and EMC (<u>Boros et al., in preparation</u>) and publish here some information ab including data about their antimicrobial potential). In this paper, we give an account of the detail of constructing our *hfq*-del mutants from our EMA and EMC [16] (Fodor et al., 2022) strains, and provide phenotypic descriptions of them. Our easyPACId strains will be available for cooperation with fellow scientists. all over the world .

2. Materials and Methods

As for "living materials", in this study plasmids, prokaryotic, and eukaryotic cellular organisms were used. Plasmids used in this study [69] (Tobias et a., 2017) , [76–84] **(see Table A1 in Appendix A)** served as molecular tools, so did some bacterium strains. Some prokaryotes (EPB cells) as antimicrobial producers. Others, like Gram-positive, and Gram-negative pathogenic clinical isolates, served as test organisms, and so did those eukaryotes (protists, fungi, and tissue culture cells) we mention.. <u>Plasmids</u> with a temperature-sensitive pSC101 replication system were growth at 30°C with antibiotic selection applied while curing these plasmids was carried out by growth at 30°C without antibiotic selection, followed by testing the resistance pattern of individual colonies for selecting plasmid-cured clones, [76] (Cherepanov and Wackernagel, 1995).. For cloning purposes, <u>*E. coli* TG1</u> **[**77] (Gibson, 1984) was used as the host strain. For plasmid mobilization into *Xenorhabdus*, <u>*E. coli* S17-1λ pir</u>   [78] (Simon, Priefer, & Pühler, 1983a) was applied. *E. coli* strains were routinely grown in LB at 37°C with appropriate antibiotic selection.

2.1. NR-AMP Producing EPB Strains, Test Organisms. Prepatatno and Biassays of CFCMs

*2.1.1.*The <u>anti-intracellular-parasite protist activities</u> of both <u>the native</u> (that is: centrifuged, and sterile-filtered (through 0.22 μm filter), and <u>heat-sterilized</u> (that is centrifuged, sterile-filtered (through 0.22 μm filter), and then (autoclaved) **CFCMs** of liquid cultures of 3 antimicrobial-producing EPB strains, EMA, EMC and EMK, representing 3 species, *Xenorhabdus budapestensis* nov. DSM16342(T) **[**15] (Lengyel et al., 2005), *Xenorhabdus szentirmaii* [15] (Lengyel et al., 2005)), and *X. innexii* nov. DSM16342(T) [15] (Lengyel et al., 2005), [15] (Lengyel, et al., 2005). [16] (Fodor, at al, 2022), were bioassayed on *Leishmania donovani* and *Trypanosoma cruzi* human pathogenic clinical isolates, as well on expectedly antibiotic-susceptible (indicator control) organisms, such as clinical isolates of Gram-positive (methicillin-sensitive (MSSA), and -resistant (MRSA) *Staphylococcus aureus*, and on Gram-negative (*Francisella novicida, Escherichia coli, Salmonella typhimurium*, and *Pseudomonas aeruginosa*) bacteria, and the commensal pathogenic fission yeast, *Candida albicans*,).The   J744 murine macrophage cells served, [59] (Geroldinger et al., 2019), as a control for measuring the general eukaryotic cytopathogenicity of the CFCMs.

2.1.2 The <u>anti-extracellular-parasite protist activities</u> of both <u>the native</u> (that is: centrifuged, and sterile-filtered (through 0.22 μm filter), and <u>heat-sterilized</u> (that is centrifuged, sterile-filtered (through 0.22 μm filter), and then (autoclaved) **CFCMs** of liquid cultures of 2 antimicrobial-producing EPB strains, *Pl.*-„Yellow" and *Pl.*-„RED"), (both grown in Medium 199 containing Earle's Salts, L-glutamine, 25 mM HEPES, L-amino acids, 15% heat-inactivated fetal bovine serum FBS and 0.22% rice starch for 65 hours at 30 °C.), representing the type strains of 1 EPB species *Photorhbdus luminescens* TT01, [85] (Waterfield t al., (2005). The latter species were represented by 2 colony-color variants (yellow and red) distinguishable on MacConkey [86] (Rahaman et al., 1975) agar plates..

2.2. Bioassays

2.2.1. Bioassays on   Leishmania and Trypanosoma Cruzi Species, as well as on Control Test-Organisms.

The bioassays were extended to clinical pathogenic protozoa using the previously described routine methods [87] (McGwire, and Kulkarni 2010). CFCM of EMA and EMC (stored in a cold room,

4 oC) for two years) were taken for these experiments. Both native and autoclaved CFCM were tested in each bioassay. Samples of EMA-CFCM and EMC-CFCM were also fractionated using Centricon spin columns as described by McGwire (unpublished) and fractions of different size ranges were assayed for activity. The bioassays were carried out in 96-hole tissue culture plates, in 200 ul volumes, containing 95 μl of the respective liquid media, 100 μl cell-free respective CFCM, and 5 μl of overnight LB cultures of the respective test organisms in duplicate. Briefly, colony count assays were used to measure activity against clinical isolates of bacteria (and Candida). Overnight incubation in 25-100 microliters of LB medium was followed by plating on a solid medium. MTT utilization assays were performed after overnight incubation of cultures up to stationary phase pro- or epimastigotes, respectively with CFCM (1:1 ratio) for 4hrs for anti-leishmanial and- trypanosomal activity. Each case's control was of untreated microbes assayed in parallel with the LB medium alone. A reduction of CFUs (for bacteria or Candida) or MTT activity (for protozoa) of at least 50% was considered + for antimicrobial activity. In our *Leishmania* studies, macrophages, macrophage culture were used as cytopathogenecity control test-organism: the macrophage cell line J774A.1 (TIB-67; ATCC, Manassas, VA, USA) was used for all present study experiments. J774 cells [62] (Tominaga et al., 1998), [63] (Andreu et al., 2017) were grown in DMEM with high glucose (25 mM) and 1.5 g/L NaHCO3, 50,000 U/L penicillin 50 mg/L streptomycin, and 10% FCS (heat-inactivated). Cells were grown in bioreactor tubes in an incubator at 37 °C and 5% CO2. The J774 cells were passaged twice a week, similar to those described in more detail later, [64] (Geroldinger et al., 2019).

2.2.2. Bioassays on.H. Meleagridis Grown in Monoxenic Culture with / E. coli

Each of the above-mentioned CFCMs were tested on *Histomona*s cells grown *in vitro* monoxenic culture with *Escherichia coli* DH5α cells.

The monoxenic culture of *Histomonas meleagridi*s Turkey/Austria/ 2922-C6/04 with *Escherichia coli* DH5α which was grown in Medium 199 containing Earle's Salts, L-glutamine, 25 mM HEPES, L-amino acids, 15% heat-inactivated fetal bovine serum FBS and 0.22% rice starch for 72 hours at 40 °C, as described before, [48.] (Ganas et al., 2012), [61] (Amin et al.,2012), [49] (Gruber et al., 2017), [51] (Bilic and Hess , 2020).

Consequently, the anti-*E.coli* activities also had to be determined. In the first experiment (see Supplementary Material) the anti-histomonal tests were cell-free filtrates from cultures of *P. luminescens* TT01 yellow and red variants grown in Medium 199 containing Earle's Salts, L-glutamine, 25 mM HEPES, L-amino acids, 15% heat-inactivated fetal bovine serum FBS and 0.22% rice starch for 65 hours at 30 °C were tested on E.coli and then the treated culture s were plated, while the effects on the *Histomonas* were measured by using a scoring system .The applied scoring system [61] (Amin et al. (2012), as well as the suggested scale we used are presented in **Table 2)**. For scoring the degree of cell destruction, the following system was applied: 0 = intact layer; 1 = up to 25% of the cell-layer destructed; 2 = 25-50% of the cell-layer destructed; 3 = 50-75% of the cell-layer destructed; 4 = more than 75% of the cell-layer destructed). In these experiments, 60% dilution of EMA-CFCM and 80% dilution of EMC-CFCM were used as stocks and serially diluted further. In the second experiment, serially diluted CFCMs were tested. For more details, see **Supplementary Material.**

2.3. Construction and Bioassay of the Antimicrobial Potential of hfq(del) Mutants of EMA and EMC

2.3.1. Construction of hfq(del) Mutants of EMA and EMC (for Detais, se Appendix A)

The Δhfq mutant EMC strain was generated according to the one-step gene inactivation method [79] (Datsenko & Wanner, 2000), which was adapted to *Photorhabdus* [80] (Yin et al., 2015) then to *Xenorhabdus.*

The Δhfq mutant EMC strain was generated as follows: The apropriately modified plasmids (As for the relevant features of plasmids, see **Table A1 in Appendix A,**) were introduced into *X. szentirmaii* (EMC) by electroporation in our lab. Electrocompetent cells were prepared for introduction of plasmids as follows: 1 ml of *X. szentirmaii* (EMC) culture grown at 30°C in LB broth supplemented with Ap (150 μg/ml) to OD600~1.1-1.2 was centrifuged for 30 s at 9000 rpm at 2°C, then resuspended in ice-cold wash buffer SMG (0.5 M sorbitol, 0.5 M mannitol, 10 % glycerol) and centrifuged again. The washing step was repeated two times, then the cells were resuspended in 60μl SMG buffer. Approximately. 1 μg plasmid DNA purified by the QIAGen Plasmid Midi Kit

according to the manufacturer's protocol was added to 40μl electrocompetent cells. Electroporation was carried out using 2-mm gap electroporation cuvettes and BTX Electro Cell Manipulator 600 with the setting 129 ohm 24 kV/cm. Transformants were selected on LB plates supplemented with the appropriate antibiotics (Km - 120 μg/ml, Sm – 100μg/ml, Gm – 50 μg/ml, and Ap - 150 μg/ml).

For the hfq KO recombination, EMC cells containing plasmid pBZs7 were grown in LB broth supplemented with Gm and Ap to a late logarithmic (OD$_{600}$~1.1-1.2) phase, then the culture was 2x diluted with fresh LB broth, L-arabinose was added in a final concentration of 1% and grown further in a shaker for 1.5 h at 30°C. The electro-competent cells were prepared as described above. The KO PCR fragment was amplified using pKD4 template plasmid and the primers hfq_EMCdelfor and hfq_EMCdelrev. The amplicon was precipitated in 96% ethanol, dried, and resuspended in 10 mM Tris pH 8.0. For electroporation, approx. 1 μg purified DNA fragment was used as described. After electroporation, cells were grown for 5 h at 30°C, then spread on LB agar plates supplemented with 120 μg/ml Km and incubated at 30°C for 2 days. The Δhfq::Km^R recombinant was tested by colony PCRs. For deletion of the Km^R cassette from the Δhfq::Km^R mutant, pBZs1 was electroporated. A Sm^{R}Ap^{R}Km^R transformant colony was grown overnight at 30°C in LB broth supplemented with Ap, spread on LB+Ap agar plates, and incubated for 24 h at 30°C, then the colonies were individually tested for the loss of the Km^R gene, and the pBZs1 plasmid on LB+Ap, LB+Km, and LB+Sm plates. The Km^S Δhfq strains were tested by colony PCR using the primer pair hfqseqfor-hfqseqrev.

The Δhfq mutant EMA strain was generated according to [69] (Tobias et al., 2017). The 769-bp upstream and the 617 bp downstream region of the hfq gene of EMA were amplified with primer pairs hfq_upforBam-hfq_uprev and hfqEMA_downfor-hfq_downrevSac, respectively. The two amplicons were assembled in the following PCR: The assembled amplicon was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), digested with BamHI and SacI, and ligated into the BamHI-SacI site of pBluescript II SK(+) (pBZS13). After sequencing, the BamHI-SacI fragment from pBZS13 was transferred into the BamHI-SacI site of the R6Kγ-based plasmid pBZs12, leading to the SacB-bearing mobilizable recombination vector pBZs17.

pBZs17 was mobilized into EMA from *E. coli* S17-1 λ pir. The ON culture of S17-1 λ pir/pBZs17 was 50× diluted with fresh LB broth supplemented with Km and Sm and grown 3 h to OD600~0.5, while the EMA recipient was grown in LB+Ap ON to OD600~1.0. 0.1 ml of donor and 1 ml of recipient cultures were mixed, centrifuged, and washed twice with 0.9% NaCl solution, then spread onto LB agar plates.

After 4h incubation at 30°C, the bacterial lawn was resuspended in 3 ml 0.9% NaCl solution, and the cells were centrifuged and spread onto LB+KmAp agar plates. After four days of incubation at 30°C, the KmRGmRApR recombinant colonies were tested by colony PCRs using primer pairs EMAhfqseqfor-trn5neo3 and pUCfor24-hfqseqrev.

To gain Δhfq recombinants, the EMA::pBZs17 strain was grown for 18 h at 30°C in LB+Ap broth, and 50, 100, and 200 μl of the culture was spread onto LB+Ap+5% sucrose agar plates. After 48h incubation at 30°C, the individual SacR colonies were tested for KmS, GmS phenotype, then, the appropriate colonies were tested by colony PCR using primers EMAhfqseqfor and hfqseqrev. To verify the *hfq* deletion, the amplicon was sequenced.

2.3.2. Bioassy of the Antimicrobial Activities of Wild-Type and hfq(del) Mutants of EMA and EMC

The bioassays were carried out in 96-hole (8 rows and 12 columns) tissue culture plates, in 200 ul volumes, containing 95 μl Mueller-Hinton liquid media, 100 μl cell-free respective CFCM, and 5 μl of overnight LB cultures of the respective test organisms in duplicate. Each test was carried out in duplicates, in the following way: rows AB: contained 100 μl wild-type CFCMs, 95 μl Mueller-Hinton (MH)-media, and 5 μl of stationery phase test organisms from 37 oC overnight cultures in each hole. Rows C and D rows contained 100 μl of the *hfq*-del mutant CFCMs of the same species (EMA, or EMC), 95 μl Mueller-Hinton (MH)-media, and 5 μl of stationery phase culture of the respective test organism (from 37 ºC overnight cultures) in each hole. Row E: 100 μl wild-type CFCMs, 100 μl Mueller-Hinton liquid (MH)-media. Row F: 100 μl the *hfq*-del mutant CFCMs of the same species (EMA, or EMC), 100 μl Mueller-Hinton (MH)-media. Row G: 195 μl of 50% Mueller-Hinton (MH)-media + 5 μl of stationery phase test organisms (from 37 ºC overnight cultures) in each hole. Row H: Row G: 200 ul of 50% Mueller-Hinton (MH)-media 5 μl of stationery phase test organisms(from 37 ºC overnight culture) in each hole.

2.4. Statistical Analysis

ANOVA was carried out using the respective propositions of the SAS 9.4, see Acknowledgment section Software mainly due to unbalanced data set. The significant differences (α = 0.05) between treatment means were assessed using the Least Significant Difference (LSD).

3. Results

3.1. Results on Anti-Leishmanial Potential

Antileishmanial activities of the cell-free conditioned media (CFCM) of three species of the entomopathogenic nematode-symbiont bacterium genus *Xenorhabdus, X. budapestensis* sp. nov (Lengyel) DSM16342 (T) EMA, *X. szentirmaii* sp. nov.,(Lengyel) DSM 16338 (T) EMC, and *X. innexii* sp. nov., (Lengyel) DSM16336 (T) EMK were determined, and compared with antibacterial and antifungal potential. Each of the three (EMA, EMC, EMK) examined CFCM showed significant anti-leishmanial activities, but EMA and EMC exerted stronger anti-microbial potential in general than that EMK. Data are summarized in **Table 1**.

Each of the three CFCMs strongly antagonized each of the tested clinical isolates of Gram-positive (methicillin-sensitive (MSSA), and resistant (MRSA)) *Staphylococcus aureus.* Similarly, these CFCMs were active against clinical isolates of Gram-negative clinical isolates of *Francisella novicida, Escherichia coli, Salmonella* Typhimurium, as well as *Pseudomonas aeruginosa*, and a clinical isolate of *Candida albicans* (**Table 1**).

Autoclaved CFCM samples of EMA and EMC were as active as the native ones. As for EMK, only native CFCM samples were tested. The results demonstrated the thermostability of the active components in CFCM of EMA and EMC since autoclaving of these samples did not affect the antimicrobial activity against any of the organisms.

As for the molecular sizes of the active natural fractions of the CFCM, interestingly the antimicrobial activity against all microbes was retained in different size fractions of EMA (below 3kDa) and EMC (above 3kDa

Table 1. Antimicrobial Activities of 5-day Mid-Stationary-Phase Cell-Free Conditioned Culture Media (CFCM) of *Xenorhabdu*s species.

	BIOCHEMISTRY			ANTIMICROBIAL ACTIVITY							TOXICITY		
				Bacteria									
CFCM	Heat	PK	Centricon	Gram +)		(Gram -)				Protist parasite		Fungus	TOX
				MS	MS	FN	ST	EC	PA	LA	TC	CA	
EMA	+	-	<3 kDa	+	+	+	+	+	+	+	-	+	
EMC	+	+	>3 kDa	+	+	+	+	+	+	+	-	+	
EMK	ND	ND	ND	+	+	+	+	+	+	+	-	+	

Table footer: Abbreviations: CM = 5-day Mid-Stationary-Phase Cell-Free Conditioned Culture Media of *Xenorhabdus* species: EMA = *X budapestensis* nov. DSM16342T; EMC = *X. szentirmaii* nov. DSM16338ᵀ; EMK = *X. innexii* nov. DSM16336T; Heat: autoclaved in standard conditions (15 min, 121oC, 1 Atm) and then tested; Centricon = indicates the molecular size of the separated fraction showing all antimicrobial activities; Tox = host toxicity, tested on determined microscopically and also determining MTT metabolism of J744-line murine macrophages (serving as checking for unspecific general cytotoxicity), exposed to the respective CM at 37oC for 72h. Abbreviations of the name of the tested microorganisms: MS and MR: methicillin-sensitive and -resistant *Staphylococcus aureus*, respectively; FN = *Francisella novicida*; ST = *Salmonella typhimurium*; EC = *Escherichia coli*; PA = *Pseudomonas aeruginosa*; LA = *Leishmania amazonensis*; TC = *Trypanosoma cruzi*; CA= *Candida albicans*. Activity assays were performed on cultured cells of the respective microorganisms incubated in 25-100 microliter of Luria Bertani-based, and appropriately supplemented media overnight. Each was then plated on the appropriate medium to CFU reduction assays (concerning bacterial or fungal pathogens) or using MTT utilization

3.2. Results on Anti-Histomonal Potential

To learn whether the NR_AMP molecules were produced by strong AMP-producing EPB species,   the antihistomonal potential CFCMs from 4 different cultures were compared, see Materials and Methods. Each CFCM was tested on *Histomonas* cells which, (as discovered by Petra Ganas el (2012) [33]) can be *in vitro* cultured reproducibly in monoxenic culture with *Escherichia coli* DH5α cells. Consequently, the anti-Gram-negative bacterial activities were also determined. As for the *Photorhabdus* data, they are presented in **Figure 1,** (effects on *Histomonas*), and Table 2 (effects on *Escherichia coli* DH5α) cells. The results indicated that there is no difference between the antimicrobia/anti-*Histomonal* potential of the colony color variants as both exerted equally strong effects on both partners of the monoxenic culture. In the cultures supplemented with either the red or the yellow colony color variants of TT01, CFCM, the cell propagation ceased and the *Histomona*s cells - earlier or later – died.
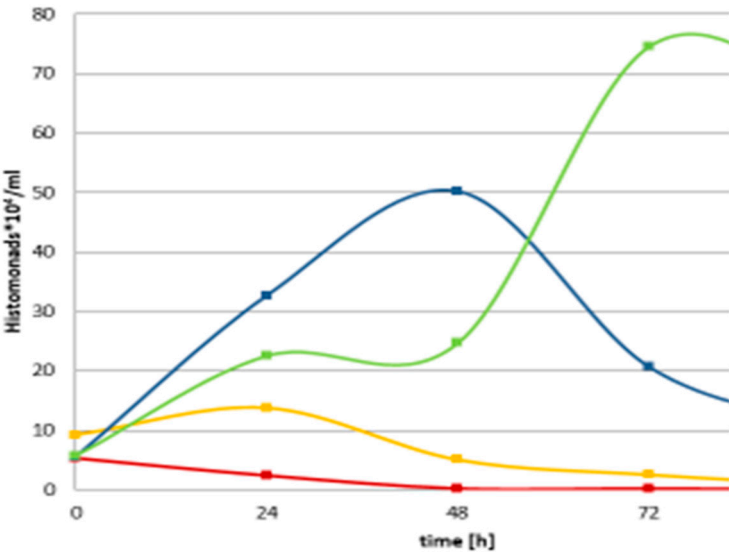

Figure 1. Monoxenic culture: H. meleagridis (Turkey/Austria/ 2922-C6/04) cells were co-cultured with E. coli DH5 @ (alpha) cells: (a) in not-supplemented M199 tissue culture media, which contained FKS (green, or (b) in supplemented M199 tissue culture media (blue) (see Materials and Methods). The cells were viable, and propagating up to   72h in (a) (green), or at least 48 hours in (b) (blue), at 40°C in the CFCM-free cultures. In those cultures however,   which were supplemented with the sterile filtered CFCM (either of the red or the yellow) TT01 colony-color variants), the cell propagation quickly ceased, both in the supplemented M199 media, (red, earlier) and in the non-supplemented (but FKS containing) M199 media, a little later) and then the Histomonas cells died. (Data are given in the Supplementary Material).

Table 2. Effect of   *Photorhabdus* CFCMs on the prokaryote partner of   *H .meleagidis* in monoxenic cuulture.

				E. coli DH5 alpha		
Sample	Hours	0	24	48	72	96
1	TT01 Yellow		Single colonies	Single colonies	Single colonies	Single colonies
2	TT01 Yellow		Single colonies	Single colonies	Single colonies	Single colonies
3	TT01 Yellow		Single colonies	Single colonies	Single colonies	Single colonies
4	TT01 Red		Single colonies	Single colonies	Single colonies	Single colonies
5	TT01 Red		Single colonies	Single colonies	Single colonies	Single colonies
6	TT01 Red		Single colonies	Single colonies	Single colonies	Single colonies
7	M199+15%MKS		Cell layer	Cell layer	Cell layer	Cell layer
8	M199+15%MKS		Cell layer	Cell layer	Cell layer	Cell layer
9	M199+15%MKS		Cell layer	Cell layer	Cell layer	Cell layer
10	Unchanged		Cell layer	Cell layer	Cell layer	Cell layer
11	Unchanged		Cell layer	Cell layer	Cell layer	Cell layer
12	Unchanged		Cell layer	Cell layer	Cell layer	Cell layer

[1] 100 ul samples had been taken from *H. meleagridis* / *E. coli* monoxenic culture (grown in M199 media), and plated on McConkey agar afterward, where only the *E. coli* cells can grow, while the *H. meleagridis* cells cannot.

From Figure 1, and Table 2 we concluded that the tested CFCM of each of the two colony-color variants of the EPB *P. luminescence* exerted cytotoxic activity not only on the prokaryotic (*E. coli* DH5 @ (alpha), but the eukaryotic (*H. meleagridis* Turkey/Austria/ 2922-C6/0 pathogen) partners as well. The growth curve of the monoxenic culture from the non-supplemented, (that is, only FKS-containing M199) control media, (green), could be characterized as performing a perfect log phase, after a moderate growth period. It starts at 24h and reaches a peak between 48–72hrs, after which it declines. The growth curve of the monoxenic culture grown in the „completely supplemented" M199 control media (blue) can be characterized by a log phase that starts right after the start of incubation (at 0 h), and then reaches a peak at 48 h, and then it rapidly declines. can be observed. Each of the exogenous EPB CFCM drastically killed the *Histomonas* cells. CFCM of the red variant acts immediately, while that of the yellow variants after 24 hrs. Even in the latter case, the size „growth peak" at 24h was less than half of the 4h value of the respective control cultures. As expected, the CFCM of each colony color variant was almost totally killing all the cells of the *EMA a and* monoxenic accompanying prokaryotic species *E. coli* DH5 alpha (**Table 2**).

3.3 . Antimicrobial Activities of the hfq(del) Mutants EMA, and EMC

We have constructed hfq-del regulatory mutants sim*ilar to th*ose of [67] (Bode et al., 2019) from both E*MA and* EMC strains. **The re**levant publication has been in preparation, the method we constructed can be seen in **Appendix A**. Wethen bioassayed the antimicrobial activities of the respective CFCMs on several targets. We intend to apply the recently discovered, and elaborated ingenious method called the easyPACId method [k1] d to identify antiprotozoal compounds in the future, similarly as it had been done by Prof. Selcuk Hazir, and his associates at Menderes University in Ankara, Turkey, [70] (Gulsen et al., 2022). his technique has been discovered and elaborated by Edna Bode and her associates in Frankfurt; Germany, [66] (Bode et al., 2019). Briefly, *Δhfq* mutants of two studied EPB bacterial species *X. budapestensis* and *X. szentirmaii were* constructed, and then the CFCMs of the wt and *hfq* mutants were used to test different pathogen organisms. But before doing that we intend to forecasts the limitations of the potential use of this approach. To learn how this fact influences the reproducibility of the method we tested the antimicrobial potential of the hfq mutant of both EMA and EMC species, and the data are presented in **Tables 3, 4**, **and 5**

Table 3. Anti-Gra-negative activities of CFCMs of wild-type n *hfq* (del) regulatory mutant cells of *Xenorhabdus budapestensis* DSM16342, and *X. szentirmaii* DSM 16338 (EMC).

TEST ORGANIS?	EMA (WT)	EMA (hfq-del)	EMC (WT)	EMC hfq-del)
GRAM NEGATIVE TARGETSS				
E. coli	-	**-**	-	+
Klebsiella pneumoniae	-	**-**	+/-	+
Salmonella enterica	-	+	+	+
Pasteurella multocida	-	**-**	+	+
Mannheimia haemolytica	-	+	+	+
Ps. aeruginosa VB(növ)	-	-/+	+	+
Actinobacillus equuli	-	+	+	+
Enterobacter cloacae	-	+/-	+	+
Serratia marcescens	-	+	+	+
Bordetella bronchiseptica	-	-	+	+

* It can be seen that each of the tested Gram-negative bacteria of veterinary significance proved completely sensitive to (one or more antimicrobial compounds in the CFCM of t wild-type *Xenorhabdus budapestensis* DSM16342 (EMA) strain. The majority of them (except for *E.coli, Salmonella*, and *Bordetella*) proved resistant to the antimicrobial active compound(s) present in the CFCM of the *hfq* (del) regulatory mutant of the same species (EMA). All but *E. coli and Klebsiella* proved)sensitive to the antimicrobial ingredient present in the CFCM of the wild-type *X. szentirmaii* DSM 16338 (EMC), but each of the proved fully resistant to the antimicrobial active compound(s) present in the CFCM of the *hfq* (del) regulatory mutant. Abbreviations: -: the test organism grew in the given CFCM, which is resistant to the antimicrobials present; +:: the test organism did not succeed in the given CFCM, which is sensitive to the antimicrobials present; +/-: the test organism grew at least one in one of the replicates, the given CFCM, that may be resistant to the antimicrobials present..

These data indicate that the strong anti-Gram-negative potential of the EMA strain severely but not probably not exclusively is under the control of the *hfq*-govern genetic regulation [68] (Tobias, et al., 2016).   The anti-Gram-positive antibacterial potential of EMC is limited in comparison to the EMA. It is similarly true concerning the anti-Gram-positive antibacterial potential, see Table 3.

Table 4. Anti-Gram-positive activities of CFCMs of wild-type n *hfq* (del) regulatory mutant cells of *Xenorhabdus budapestensis* DSM16342, and *X. szentirmaii* DSM 16338   (EMC).

TEST ORGANISMS	EMA (WT)	EMA (*hfq*-del)	EMC (WT)	EMC *hfq-del*)
GRAM POSITIVE				
Bacillus cereus	-	-	-	NT
Staphylococcus pseudintermedius	-	-	+	NT
Staphylococcus aureus (MRSA)	-	-	+	NT
Staphylococcus hyicus	-		-	NT
Streptococcus suis	-	+	-	NT
Streptococcus agalactiae	-	-	-	NT
Listeria monocytogenes	-	+	-	NT
Erysipelothrix rhusiopathiae	-	-	-	NT
Corynebacterium pseudotuberculosis	-	-	-	NT
Rhodococcus equi	-	-	-	NT

* It can be seen that each of the tested Gram-positive bacteria of veterinary significance is completely sensitive to (one or more antimicrobial compounds in the CFCM of t wild-type *Xenorhabdus budapestensis* DSM16342 (EMA) strain. All but two 2 (*Streptococcus suis*, and *Lysteria monocytiogene*) Gram-positive test bacteria proved also susceptible to the antimicrobial active compound(s) present in the CFCM of the *hfq* (del) regulatory mutant of the same species (EMA). All but (Uaniher) two Gram-positive bacteria (*Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius*)sensitive to the antimicrobial ingredient present is the CFCM of the wild-type *X. szentirmaii* DSM 16338 (EMC). Abbreviations: -: the test organism grew in the given CFCM, which is resistant to the antimicrobials present; +:: the test organism did not succeed in the given CFCM, which is sensitive to the antimicrobials present; +/-: the test organism grew at least one in one of the replicates, the given CFCM, that may be resistant to the antimicrobials present.

These data indicate that the extremely strong anti-Gram-positive potential of the EMA strain severely but probably not exclusively is under the control of the *hfq*-govern genetic regulation [68] (Tobias, et al., 2016).   The anti-Gram-positive antibacterial potential of EMC is stronger than the anti-Gram-negative potential, but, in comparison to that of the EMA is weaker. Note that the EMC-resistant Gram-positive species are not the same that those which are resistant to the antimicrobials present in the CFCM of the *hfq*-del regulatory mutant of EMA. To see how the intraspecific variability modifies the picture, we compared the resistance/sensitivity patterns of different geographical isolations of the unambiguously EMA,-sensitive Gram-positive, Paeibacillus larvae [88] (Ebeling et l., 2016). bacterium species. The *P. larvae* a rapidly spreading deadly pathogen organism for honey bee larvae are a significant pest of agricultural significance.

Table 5. Intraspecific variability within the Gram-positive *Paeibacillus larvae* concerning semsitivities toward antimicrobial active compound of the CFCM of wild-types hfq (del) regulatory mutants strains of *Xenorhabdus szentirmaii* (EMC) EPB species**.**

TEST ORGANISMS	EMA (WT)	EMA (*hfq*-del)	EMC (WT)	EMC *hfq-del*)
GRAM POSITIVE				
Paenibaillus larvae isolates				
ML-234	-	**+**	+	+
ML-236	-	**+**	+	+
ML-237	-	**+**	+	+
ML-238	-	**-**	+	+
ML-239	-	**+**	+	+
ML-248	-	-	-	+
ML-250	-	-	-	+

ML-252	-	-	-	+
ML-263	-	-	-	+

*It can be seen that each of the tested geographical isolates of the Gram-positive, (deadly pathogen for honeybee larvae) *Paeibacillus larvae* [88] (Ebeling et l., 2016) bacterium species seems to be completely sensitive to (one or more) antimicrobial compounds present in the CFCM of t wild-type *Xenorhabdus budapestensis* DSM16342 (EMA). Six of the nine isolates, however, seem also to be sensitive to the antimicrobial active compound(s) present in the CFCM of the *hfq* (del) regulatory mutant of the same species (EMA). About half of them (mainly those which are resistant to the antimicrobials of the *hfq* (del) regulatory EMA mutant) proved also resistant to the wild-type EMC CFCM., but each of them is resistant to the antimicrobials present in the CFCM of the *hfq* /del) a regulatory mutant of EMC.

These data indicate that although the *Paeibacillus larvae* species are uniformly, and unambiguously se**n**sitive to the antimicrobial(s) present in the CFCM of EMA wild-type strain, there is a significant intraspecific variability concerning partly the resistance//sensitivity issue to EMC-produced antimicrobials, partly resistance/sensitivity issue to the antibiotic potential if EMA hfq-del regulatory mutants. These data are of key importance when drawing our conclusion concerning the usefulness and the limitation of the potential use of the easyPACId [71] (Bode et al., 2019) approach in our future research. These data also confirm that the extremely strong anti-Gram-positive potential of the EMA strain severely but probably not exclusively is under the control of the *hfq*-govern genetic regulation [68] (Tobias, et al., 2016). As for our anti-histomonal stufies, we follow this way..

In (Figure 2 one can see the difference between the colonies of the wild. type and the *hfq*-(the regulatory mutant of the *X. szentirmaii* DSM 16338 (EMC). The spectacular difference is the presence (in the wild-type, left, a) and the mutant (right, b)

(**A**)   (**B**)
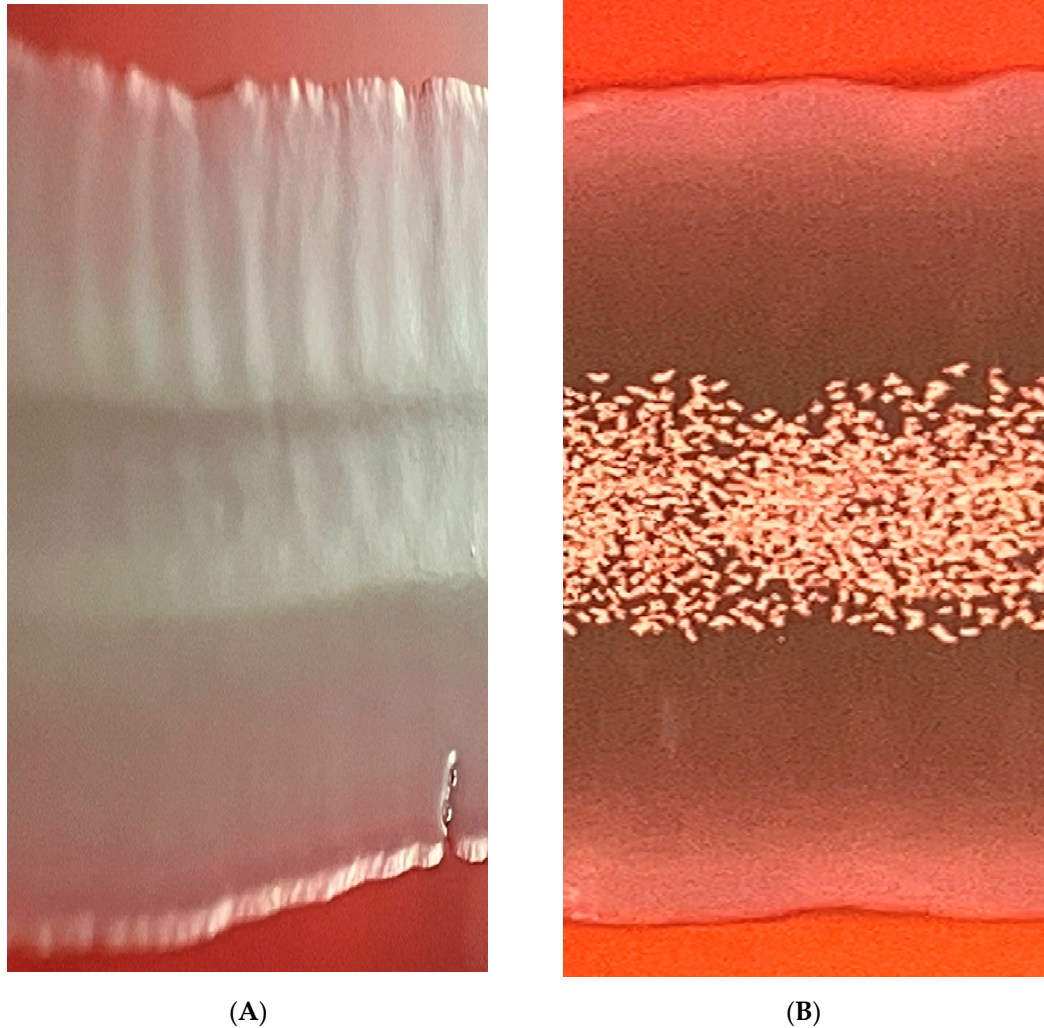
Figure 2. Colony of the wild-type (left, B, and down, C), and the *hfq*-del regulatory mutant (left, A) of *Xenorhabdus szentirmaii* DSM16339 (EMC). The spectacular iodinine crystals, (the end product) of the phenazine biosynthesis are missing, indicating that the phenazine-operon encoded biosynthesis pathway is switched off. in the hfq-deleted regulatory mutant.

4. Discussion.

This paper is about the anti-protist potential of antimicrobial compounds produced by entomopathogenic bacterium species (EMA, EMC, EMK *Photorhabdus luminexscens* TT01 [15] (Lengyel et al.,2005; [16] Fodor et al., 2022); Fodor et al.,2023, [85] (Waterfield, 2005) the obligate symbionts of certain entomopathogenic nematodes, (EPN), into their conditioned culture media (CFCM) *in vitro,* As for their chemical nature, of these antcrovials, they are non-ribosomal antimicrobial peptides, (NR-AMPs) which are enzymatically synthetized through more than one steps, in their respective biosynthetic pathways. Those genes encoding for the enzymes forming the respective biosynthetic pathway of a given NR-AMP are clustered in a single operon [92] (Jacob et al., 1960), also coalled as biosynthetic gene complex (BGC), [93] (Gualtieri et al., 2022),   .One of the natural role of the procryiote partner in the EPN/EPB symbiosis is to protect of the given monoxenic symbitic association in a given polyxenic milieu, producing a chemical toolkit (e.g. a particular set of NR-AMPs) providing competitiveness against the prokaryotic and eukaryotic competitors, including protists. The biosynthesis of the given sets set of these effective antimicrobials are synchronized by unique, genuine hierarcchical   genetic regulation mechanism called primary-secondary phase shift [62] (Nealson and Forst, 1996) with the *hfq* gene on the top, [66–70]. In laboratory liquid culture conditions (as well is in the active symbiotic stage) these compounds are produced abundantly providing an unexhausstible   "golden mine" to the antimicrobial searching scientist, The bioassays of the CFCMs are the tools find e best sources and optimize the culture conditions, We had found that those strains (EMA and EMC) which were discovered and further developed not only by our international team of a laboratory without wallbot in those famous laboratories as those of Maxime Guatier in France and Helge Bode in Germany   proving that these two strains are probably best what have eves found so far. and other laboartories are probably really excellent sources. These modest data presented here on an anti-Leishmanial and anti-Histonal potentials are seem to confirm this conclusion. The next step is to find a way to get   mutant (not "genetically manipulated", but mutant strains which are produce only single NR_AMP molecules instead of the banch of them. Te method to do that was recently discovered in the Bode laboratory [71–75] (Bode et al., 2019). We demonstrated heret hat we have strarted this project as well by constructing and characterizing hfq-del mutants from both EPB species EMA, and EMC). Our goals is to targeting to pathogens of veterinary and agricultural significance.

We have been interested in the drug potential of the NR-AMP of EMA and EMC   against eukaryotic parasites and pathogens of eukaryotic (mammal and bird) hosts. We wonder whether a chemotherapeutic approach based on applying   *Xenorhabdus* NR-AMPs to prevent and controlling of flagellate parasitic pathogen protozoan species like the intracellular mammalian (and human) pathogenic *Leishmania donovani* (Laveran & Mesnil, 1903) species complex, [25] (Fernández-Arévalo et al., 2020) and the extracellular avian pathogenic *Histomonas meleagridis*, (Smith, 1895), [89] (Gerbod et al., 2001), are a realistic possibility. Both target species are flagellated protozoon, however, *Leishmania* is intracellular, while *Histomonas* is an extracellular parasite, and both own strong, unique, and efficient resistance mechanisms. The strategy for targeting antimicrobials to the prokaryotic or eukaryotic parasite is entirely different because of the similarities between the intracellular structures of eukaryotic hosts and the eukaryotic parasites (pathogens). Our research conception is that we suppose that some *Xenorhabdus* NR_AMP (phenazine, fabclavine, or something else) may be capable of dysregulating mitochondrial function and stimulating apoptosis, [90] (Rochford et al., 2020).

Leishmania ssp. is one of the most problem-causing pathogens considering its sophisticated resistance mechanisms. Still, at the same time, it is an excellent model for analyzing the mechanisms of drug resistance in eukaryotes [39] (Segovia 1994) since 1956 when the first cases of clinical resistance have been appeared in [91] (Ponte-Sucre et al., 2017). From an application point of view, the medical or veterinary perspectives of natural compounds are at stake several aspects have to be taken into consideration: A) the antimicrobial potential; (B) the durability, thermotolerance, and bioavailability; (C) cytotoxicity, and unwanted side effects, especially if one group of target organisms are of eukaryotic (protist) pathogens.

We have demonstrated that *Xenorhabdus* CFCM has broad-spectrum antimicrobial activity against several - human microbial pathogens. However, it is unclear what the precise nature of the antimicrobial effects is. In other terms, the question is whether or if the same active molecules are

responsible for the killing of each organism or just a special group of organisms. The CFCMs of all three *Xenorhabdus* species proved active against *Leishmania* promastigotes in our experiments, but neither of them was efficient against not *Trypanosoma cruzi* epimastigotes. Other researchers [75] (Gulsen et al., 2022) found that EMA and EMK CFCMs were equally efficient against both *Leishmania* and Trypanosoma. They stated that *X. budapestensis, X. cabanillasii, X. hominickii, X. indica, X. innexii*, and *X. stockiae* supernatants caused 100% mortality at the highest tested centration (10%) against the promastigote form of *L. tropica*. No differences occurred between this treatment group and positive control (P > 0.05). (N-methyl meglumine was used as positive control). They did not publish data on EMC in the quoted article.

The number of alternative explanations of these discrepancies between the data of the Hazír and those of our team, maybe more than one. One of the explanations may be related to the differences in the surface compositions between the two parasites that either confer resistance to the active compounds or the inactivated penetration intracellularly to act on specific molecular targets.

The identification of the antimicrobial component(s) present in the CFCMs is important to understand, as well as their mechanisms of action. Another possible alternative explanation is that the CFCM of the *hfq*-del mutants used in the experiments in Ankara exerted some antagonistic effects on *Trypanosoma.*

We decided to compare the antimicrobial potential of each of the used *hfq*-del mutants before using it as the source of double mutants producing single NR-AMPs. We focus only on EMA and EMC species. and Our data in **Tables 35** suggest to beware of the potential basic antimicrobial activities of the *hfq*-del mutants because they may have unexpected antimicrobial potential. It would not be a surprise considering that several previous studies of different labs have revealed that approximately 7.5% of the total genes in *Xenorhabdus* bacteria are dedicated to secondary metabolite biosynthesis, and probably that majority of these products are NR-AMPs.

Calculating with about an average of 2.4Mb-size *Xenorhabdus* genomes, 1 kb locus-size, and about 10 ORF per operon, then we have to think about 2,400 genes and 240 respective operons (BGS) per genome. It compares with the average genome of *Staphylococcus*, we may count on about 225, operons. In EMA we are interested in 3, and in EMC and EMC in 12 operons only, and we cannot rule out that are several other operons encoding for enzymes to synthesize compounds with antimicrobial potential. We intend to establish and analyze RNA-seq from both the wild-type and the *hfq*-strains.

However, we suppose that the situation is probably somewhat simpler since the *hfq* probably regulates a well-defined group of operons providing the genetic background of the so-called "primary-secondary phase shift" [63] (Akhurst, 1980), ("phenotypic phase variation", the term of Professor S. A. Forst) scenario. That is, only a relatively small set of natural product (NP) genes are involved, and most of the antimicrobial-synthetic genes are among them.

Toxicity studies using J774 macrophages [57] (Tominaga, 1998), [58] (Andreu et al., 2017) indicated that only the CFCM of EMA was able to cause significant killing of these cells. In contrast, the CFCMs of EMC or EMK had no effect. This experiment confirmed our previous unpublished observations that EMA exerts stronger cytotoxicity on a range of eukaryotic cells, which may cause limitations concerning future medical applications. But this selective cytotoxicity may provide an option to select for and find specific CFCMs against different eukaryotic parasites of different eukaryotic (animal, plant) hosts.

To put the right place the significance of our anti-leishmanial results, we have to realize that the current chemotherapies used to handle these dangerous diseases have rather limited efficacy and Sb-resistance [94] (Kazemi-Rad et al., 2013) has ben spreading. As for the future, the main trend may be based upon the complete genome sequencing of multiple strains, taking advantage of the application of CRISPR/Cas9 technology, and *in vivo*, bioluminescence-based imaging has set the stage for advancing target-based drug discovery, [32] (Altamura et al., 2022). But, new drugs are urgently needed anyway.

As for *Histomonas* experiments, we concluded that in different degrees, but each studied EPB CFCM exerted rather strong cytopathogenic effects on the monoxenic culture of *H. meleagridis* Turkey/Austria/ 2922-C6/04 cells co-cultured eukaryotic targets as well, similarly to the *Eimeria* (data not shown). Considering the cytopathogenic effects were also unambiguously detected on a confluent monolayer of LMH cells (permanent chicken hepatocarcinoma liver cell

line) [21] (Fodor et al, 2023), we have to work on finding a CFCM with strong antihistomonal, and low cytotoxic activity. To do that we intended to compare the anti-histomonal, and cytotoxic potential of the wild, the hfq-delated, and different single antibiotics.-producer double mutants (hfq-del, and reactivated induced promoter-carrying single antibiotic-producer EMA and EMC strains. Considering the estimated selective cytotoxic potential of different strains we intend to use not only manganous but normal cell lines in the cytotoxicity studies. As mentioned in the Introduction, *in vitro* assays relying solely on non-phagocytic cell models may not represent a realistic result as the effect of an antileishmanial agent should ideally be presented based on its cytotoxicity profile against reticuloendothelial system cells. [59]. (Brioschi et al., 2022).

As for the results in the control (bacterium, fungi and other strains), previous studies have identified several anti-microbial molecules in the CFCM of *Xenorhabdus,* including fabclavine, [12] (Fuchs et al., 2012). [13] (Fuchs et al., 2014) a specialized metabolite polypeptide/polyketide hybrid molecule that is synthesized via non-ribosomal peptide synthesis. Many different-sized derivatives of fabclavines can be produced by the *X. szentirmaii* species [72] (Wenski et al., 2019), and can vary between entomopathogenic nematode-symbiont (*Xenorhabdus, Photorhabdus*) bacterium species [72] (Wenski et al., 2019). These have a wide-ranging anti-microbial activity against various microbes [16] (Fodor, et al., 2022).

Multiple other antimicrobial molecules can also be produced in CFCMs, including xenofuranone A and B, [95] (Brachmann, et al., 2006) cabanillasin [96] (Houard, et al 2013), PAX peptides [97 (Fuchs et al., 2011), odilorhabdins, [98] (Racine, and Gualtieri, 2019), [93] (Lanois-Nouri et al., 2022) xenematides [99] (Xi et al, 2019), anti-oomycete peptides [100] (Xiao et al., 2012)¸ xenortide [101] (Reimer, 2014), rhabdoopeptided/xenortide-like peptides, [102] (Zhao, 2018), cyclic depsipeptide xenematide F, and G rhabdopeptides [103] (Bi et l., 2018), szentiamids [104] (Ohlendorf, et al., 2011), [105] (Nollmann, et al, 2012). These latter peptides have earlier been published to be active against *Trypanosoma brucei*, but we cannot confirm that.

Whether any of them, can be considered as a potential antihistomonal drug candidate will be learned if we had a chance to screen both the single mutant *hfq*-del stains of EMA and EMC and those double mutants (3 from EMA and 11 of EMC) in which the *hfq* is deleted end only one single operon is activated

5. Conclusions

The recently discovered hierarchical regulatory systems following signal integration provides a great metabolic versatility,   that results in excellent adaptability and metabolic optimization in antibiotic- producing bacteria [106] (Monteagudo-Cascales et al., 2022). like EPBs. To emphasize the recent importance of these researches, we have to conclude that he delineation of specific anti-microbial products in CFCM can now be achieved using the "easyPACId" approach (easy Promoter Activated Compound Identification) which takes advantage of the alteration of regulation of coordinate gene clusters which products are responsible for their biosynthesis [71] (Bode et al., 2019). This discovery may revolutionize the *Xenorhabdus*/*Photorhabdus* researches, which in the age of multidrug resistance maybe important.

The point is that different biosynthetic gene clusters (BGCs, that is operons) are coordinately regulated [68] (Tobias et al., 2016) [69] (Tobias et al., 2017),by the gene encoding the RNA chaperone, Hfq, [671 (Bode et al., 2019). The experimental system is a deletion mutant of the regulatory gene Hfq [107–111] of the respective species. This kind of regulatory mutant strain does not produce any biosynthetic AMP, but the respective individual promoters upstream of the respective biosynthetic gene cluster can be changed to inducible promotors and this recombinant strain will produce only a single respective AMP molecule. This is further facilitated by the completed genome sequencing of several Xenorhabdus species including *X. szentirmaii* [102]) (Gualtieri et al., 2014), and *X. budapestensis*, [113] (Li et al., 2021) making possible the identification of the promoter regions of each biosynthetic gene cluster (BGC, operon). Creating bacterial mutants that synthesize individual antimicrobial products into CFCM can facilitate their identification purification and bioassays of antimicrobial activity. [74] Ozkan, et al., 2019), [75] (Cimen et al., 2021).

Appendix A

Expeimental details of the Construction of $hfq(del)$ *mutants of EMA and EMCsttains* .

The Δhfq mutant EMC strain was generated according to the one-step gene inactivation method **[107]** (Datsenko & Wanner, 2000), which was adapted to *Xenorhabdus*. The λ Red recombinase was replaced by the *Photorhabdus luminescence*-derived homolog encoded by the Plu2934/Plu2935/Plu2936

operon **[108]** (Yin et al., 2015). The plasmid pSC101-BAD-plu2934/2935/2936-gamma-amp containing a thermosensitive pSC101-derived replication system and the Plu2934/Plu2935/Plu2936 operon with the λ Redγ gene under the control of ParaBAD was a kind gift from Jun Fu. Plasmids were introduced into *X. szentirmaii* (EMC) by electroporation. Electrocompetent cells were prepared for introduction of plasmids as follows: 1 ml of *X. szentirmaii* (EMC) culture grown at 30°C in LB broth supplemented with Ap (150 μg/ml) to OD600~1.1-1.2 was centrifuged for 30 s at 9000 rpm at 2°C, then resuspended in ice-cold wash buffer SMG (0.5 M sorbitol, 0.5 M mannitol, 10 % glycerol) and centrifuged again. The washing step was repeated two times, then the cells were resuspended in 60μl SMG buffer.

Approx. 1 μg plasmid DNA purified by the QIAGen Plasmid Midi Kit according to the manufacturer's protocol was added to 40μl electrocompetent cells. Electroporation was carried out using 2-mm gap electroporation cuvettes and BTX Electro Cell Manipulator 600 with the setting 129 ohm 24 kV/cm. Transformants were selected on LB plates supplemented with the appropriate antibiotics (Km - 120 μg/ml, Sm – 100μg/ml, Gm – 50 μg/ml, and Ap - 150 μg/ml).

For the hfq KO recombination, EMC cells containing plasmid pBZs7 were grown in LB broth supplemented with Gm and Ap to a late logarithmic (OD$_{600}$~1.1-1.2) phase, then the culture was 2x diluted with fresh LB broth, L-arabinose was added in a final concentration of 1% and grown further in a shaker for 1.5 h at 30°C. The electro-competent cells were prepared as described above. The KO PCR fragment was amplified using pKD4 template plasmid and the primers hfq_EMCdelfor and hfq_EMCdelrev. The amplicon was precipitated in 96% ethanol, dried, and resuspended in 10 mM Tris pH 8.0. For electroporation, approx. 1 μg purified DNA fragment was used as described. After electroporation, cells were grown for 5 h at 30°C, then spread on LB agar plates supplemented with 120 μg/ml Km and incubated at 30°C for 2 days. The Δhfq::Km^R recombinant was tested by colony PCRs using the primer pairs hfqseqfor-trn5neo5, rn5neo3-hfqseqrev, and hfqseqfor-hfqseqrev.

For deletion of the Km^R cassette from the Δhfq::Km^R mutant, pBZs1 was electroporated. A Sm^{R}Ap^{R}Km^R transformant colony was grown overnight at 30°C in LB broth supplemented with Ap, spread on LB+Ap agar plates, and incubated for 24 h at 30°C, then the colonies were individually tested for the loss of the Km^R gene, and the pBZs1 plasmid on LB+Ap, LB+Km, and LB+Sm plates. The Km^S Δhfq strains were tested by colony PCR using the primer pair hfqseqfor-hfqseqrev.

<u>The Δhfq mutant EMA strain was generated according to</u> [109] (Tobias et al., 2017). The 769-bp upstream and the 617 bp downstream region of the hfq gene of EMA were amplified with primer pairs hfq_upforBam-hfq_uprev and hfqEMA_downfor-hfq_downrevSac, respectively. The two amplicons were assembled in the following PCR:

Using 0.5 μl of the upstream and 2 μl of the downstream amplicons, 60 s denaturation at 94 °C followed by 15 cycles of 94°C 20 s, 55°C 30 s, 72°C 2 min, then 0.5 μl of primers hfq_upforBam and hfq_downrevSac (10 μM each) were added and the cycling was continued for further 35 cycles. The amplicon was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), digested with BamHI and SacI, and ligated into the BamHI-SacI site of pBluescript II SK(+) (pBZS13). After sequencing, the BamHI-SacI fragment from pBZS13 was transferred into the BamHI-SacI site of the R6Kγ-based plasmid pBZs12, leading to the SacB-bearing mobilizable recombination vector pBZs17.

pBZs17 was mobilized into EMA from *E. coli* S17-1 λ pir. The ON culture of S17-1 λ pir/pBZs17 was 50× diluted with fresh LB broth supplemented with Km and Sm and grown 3 h to OD600~0.5, while the EMA recipient was grown in LB+Ap ON to OD600~1.0. 0.1 ml of donor and 1 ml of recipient cultures were mixed, centrifuged, and washed twice with 0.9% NaCl solution, then spread onto LB agar plates. After 4h incubation at 30°C, the bacterial lawn was resuspended in 3 ml 0.9% NaCl solution, and the cells were centrifuged and spread onto LB+KmAp agar plates. After four days of incubation at 30°C, the KmRGmRApR recombinant colonies were tested by colony PCRs using primer pairs EMAhfqseqfor-trn5neo3 and pUCfor24-hfqseqrev.

To gain Δhfq recombinants, the EMA::pBZs17 strain was grown for 18 h at 30°C in LB+Ap broth, and 50, 100, and 200 μl of the culture was spread onto LB+Ap+5% sucrose agar plates. After 48h incubation at 30°C, the individual SacR colonies were tested for KmS, GmS phenotype, then, the

appropriate colonies were tested by colony PCR using primers EMAhfqseqfor and hfqseqrev. To verify the hfq deletion, the amplicon was sequenced.

Restriction enzymes and DNA polymerases were purchased from Thermo Scientific. Colony PCRs were carried out using Dream Taq polymerase, while for the amplification of fragments for cloning or KO experiments, a Phusion polymerase was applied.

For maintaining the plasmids with a temperature-sensitive pSC101 replication system, growth at 30°C with antibiotic selection was applied,while curing these plasmids was carried out by growth at 30°C without antibiotic selection, followed by testing the resistance pattern of individual colonies for selecting plasmid-cured clones.

For cloning purposes, *E. coli* TG1 (Gibson, 1984) was used as host strain. For plasmid mobilization into *Xenorhabdus*, *E. coli* S17-1λ *pir* (Simon, Priefer, & Pühler, 1983a) was applied. E. coli strains were routinely growth in LB at 37°C with appropriate antibiotic selection.Restriction enzymes and DNA polymerases were purchased from Thermo Scientific.Colony PCRs were carried out using Dream Taq polymerase, while aplification of fragments for cloning or KO experiments, Phusion polymerase was applied.

Relevant features of plasmids used in this work

Name	Relevant features	References
pBluescript II SK (+)	pMB1-based ApR cloning vector.	(Short, Fernandez, Sorge, & Huse, 1988)
pKD4	Km^R,Ap^R r6kγ-based PCR template plasmid for one-step recombination gene-KO	(Datsenko & Wanner, 2000)(Datsenko & Wanner, 2000)
pBZs1	Sm^R,Cm^R,Ap^S, derivative of pCP20 (Cherepanov & Wackernagel, 1995) containing also the *oriT* of RP4, thermo-inducible FLP recombinase expression (λ pR::FLP), temperature-sensitive pSC101 replication system, λ cI857, mobilization by S17-1λ *pir* host strain	this work
pBZS7	Km^R,Ap^S derivative of pSC101-BAD-plu2934/2935/2936-gamma-amp (Yin et al., 2015), L-ara-inducible expression of λ Red recombinase homologue from *P. luminescens* and λ Redγ protein, temperature-sensitive pSC101 replication system	this work
pBZs12	pJQ200-SK derivative, where the SspI-NheI fragment carrying the p15A origin of replication was replaced by the ClaI-NheI fragment of pSG76-K containing the Km^R gene and R6Kγ origin of replication. KmR, GmR, RP4 oriT, R6Kγ oriV, SacB	this work
pBZs17	pBZs12 derivative, containing the assembled upstream and downstream region of the hfq gene of EMA. KmR, GmR, RP4 oriT, R6Kγ oriV, SacB	this work
pSG76-K	R6Kγ-based Km^R replicon	(Pósfai, Koob, Kirkpatrick, & Blattner 1997)
pJQ200-SK	A SacB-bearing p15A-based vector. GmR, RP4 oriT, p15A oriV, SacB.	(Quandt & Hynes, 1993)

Relevant Features of bacterial strains used.

TG1	supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB+ lacIq lacZΔM15]	(Gibson, 1984)

S17-1 λpir	S17-1 λpir, a λ lysogen derivative of S17-1 (pro thi recA hsdR (r⁻ m⁺) TpR SmR KmS [Ω RP4-2-Tc::Mu-Km::Tn7]) expressing Π protein from pir gene of R6K	(Simon, Priefer, & Pühler, 1983b)
X. budapestensis DSM16342	wt isolate	Lengyel, et al., 2005
X. szentirmaii DSM 16338	wt isolate	

List of oligonucleotides used.

Primer	Sequence (5'-3')	Reference
hfq_EMCdelfor	gaaccaaaaggttcttagttaaaacaacaaaataaggaaaatatagaatgGTGTAGGCTGGAGCTGCTTC	this work
hfq_EMCdelrev	aagtctccctacctatgtttttatttacttcaggtatggtcactgatttaCATATGAATATCCTCCTTAGTTC	this work
hfqEMAseqfor	tgtggtcttatctcgcgggc	this work
hfqseqfor	tctcatgatgagatggtttatcgtg	this work
hfqseqrev	gcacaggagaaacacctgcgg	this work
trn5neo3	tcgccttctatcgccttcttga	this work
trn5neo5	aatagcctctccacccaagcgg	this work
hfq_upforBam	aaggatccactcctggttggcggaaccatgc	this work
hfq_uprev	caaagattgcccttagccattctatattttccttattttg	this work
hfqEMA_downfor	caaaataaggaaaatatagaatggctaaggggcaatctttgatcgctgaataaatcagtgataatgcctgg	this work
hfq_downrevSac	aagagctctttgtcacgcagcatacggcggtc	this work
pUCfor24	cgccagggttttcccagtcacgac	(Nagy, Szabó, Hegyi, & Kiss, 2021)

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