Agrobacterium may be used as a suitable experimental system for genetic analysis of resistance to (at least *Xenorhabdus budapestensis*) antimicrobial peptide complexes

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Background Antimicrobial compounds released by the entomopathogenic nematode-symbiont bacterium *Xenorhabdus budapestensis* (EMA) are oligopeptides and the "trump" is fabclavine. They kill antibiotic multi-resistant *Escherichia coli, Salmonella*; mastitis-isolate *Staphylococcus aureus, E. coli* and *Klebisella pneumoniae*; *S. aureus* MRSA strain; plant-pathogenic *Erwinia amylovora*; *Xanthomonas*, *Clavibacter,* and *Pseudomonas* strains. Each tested *Phytophthora* isolate proved also sensitive. Fabclavine was claimed toxic, however, *Proteus*, some *Pseudomonas* and *Agrobacterium* strains are resistant. Our **goal** is to establish a suitable system for genetic analysis of antimicrobial peptide (AMP)-resistance by beneficially using the experimental toolkit of *Agrobacterium r*esearch.

Methods. We tested the anti-*Agrobacterium* activity of the native cell-free culture media (CFCM) of EMA by agar diffusion assay. EMA_PF2 peptide fraction (of reproducible HPLC and MALDI profile) was then isolated from CFCM of EMA and exerted strong AMP activity on both Gram-negative and positive targets. The sensitive/resistant (S/R) phenotype of *Agrobacterium* strains of known genotype to EMA_PF2 was determined in liquid culture bio-assays.We tested 1 wild-type (A281) and 3 T-DNA-deleted (AGL1, EHA105, A4T) agropine (L, L,-succinamopine, AGR) catabolizing strains with C58 chromosome and of pTiBo542 plasmid; 5 pTi58-plasmid-cured (HP1836, HP1840, HP1841, HP1842, HP1843) and 1 T-DNA deleted and binary vector harboring (SZL4) nopaline-catabolizing strains of C58 chromosome; and 2 T-DNA deleted octopine-catabolizing (OCT) strains with and without binary vector of Ach5 chromosome (SZL2 and HP 1837, respectively).

Results. *Agrobacterium tumefaciens* A281, HP1836, HP1840, HP1841, HP1842, HP1843, SZL4 and SZL2

proved resistant; HP1837, AGL1, EHA105 and A4T strains were sensitive to EMA PF2. All but SZL4 showed the same S/R phenotype to CFCM and EMA_PF2.

Discussion. There are both sensitive and resistant strains of C58 and Ach5 chromosome and of different opine type strains. All but one T-DNA(-) strains (SZL2) were sensitive to EMA PF2. All plasmid-cured strains and the wild-type A281 were resistant.

Conclusions. We consider EMA_PF2 as a natural complex of interacting AMP molecules and identified resistant (R) and sensitive (S) *Agrobacterium* strains to it. The S/R phenotype seems independent on both the chromosome and the opine-type. Each tested T-DNA-Deleted pTiBo542 harboring strain proved sensitive while that of harboring intact plasmid was fully resistant. The availabilities of the T-DNA-Deleted EMA_PF2 (S) and the of the T-DNA-Non-Delated EMA_PF2 (R) pTiBo542 plasmid harboring *Agrobacterium* strains may provide a suitable system for genetic (complementation) analysis for resistance mechanisms towards EMA_PF2 and maybe towards other AMPs active on Gram-negatives. The main argument is the exceptional unique opportunity for applying the genuine tools binary vector strategy.

Agrobacterium: a suitable experimental system for genetic analysis of resistance to (at least *Xenorhabdus budapestensis*) antimicrobial peptide complexes

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ABSTRACT

Background: Antimicrobial compounds released by *Xenorhabdus budapestensis* (EMA) are oligopeptides. The "trump" is the fabclavine. They kill *S. aureu*s MRSA; antibiotic multi-resistant *Escherichia coli, Salmonella*; mastitis-isolate *Staphylococcus aureus, E. coli* and *Klebisella pneumoniae*; *Erwinia amylovora*; *Xanthomonas*, *Clavibacter,* and several *Pseudomonas* strains. Each *Phytophthora* proved also sensitive. Fabclavine was claimed toxic, however, some *Proteus*, *Pseudomonas* and *Agrobacterium* strains are resistant. Our **goal** is to establish a suitable system for genetic analysis of antimicrobial peptide (AMP)-resistance by beneficially using the experimental toolkit of *Agrobacterium* research.

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other AMPs active on Gram-negatives. The main argument is the exceptional unique opportunity for applying the genuine tools binary vector strategy.

Key words: *Agrobacterium*; Ti plasmid; Intact/Cured/T-DNA Deleted; Sensitive/Resistant to; *Xenorhabdus budapestensis* / EMA; Antimicrobial peptides /AMP; EMA_PF2

INTRODUCTION

The emergence of antibiotic multi-resistance in pathogenic bacteria has become alarming in the recent decades, all over the world; invoking an enormous public concern, not only from human human-clinical,

The number of pathogenic bacterium species of clinical, (*Talbot et al.,2006; Talbot, 2008; Dötsch et al., 2009; Cantas et al, 2013*; *Exner et al., 2017*); veterinary (*Gebreyes and Thakur 2005; Endimiani et al., 2011; Szmolka & Nagy, 2013; Moore et al., 2013; Davis et al., 2013; McManus et al, 2015; Rzewuska et al., 2015; Marques et al., 2016*); and plant medical (*Załuga et al., 2014*; *Li, Plésiat and Nikaido, 2015*; *Fodor et al., 2012; Fodor at al., 2017*) aspects has dangerously been increasing. Those bacterium species which have been put in the ESKAPE (based on the initials of respective genus name) list (Rice, 2008) are: *Enterococcus faecium*, (*Williamson et al., 1983; Sun et al., 2012; Gilmore, Lebreton, & van Schaik, W. 2013; Miller, Munita &Arias, 2014*) *Staphylococcus aureus*, (MRSA) [41] (*Tomasz, 1998; Tenover et al., 2008; Ellington et al, 2010; Shi et al., 2014*); *Klebsiella pneumoniae*, (*Schechner et al., 2008; Schwaber et al., 2011*); *Acinetobacter baumannii*, (*Vila, Martí, and Sanchez-Céspedes, 2007; Antunes, Visca and Towner 2014*); *Lee et al., 2017*); *Pseudomonas aeruginosa*, (*Nordman et al., 1993; Strateva and Yordanov, 2009; Hirata et al, 2002*; *Nehme and Poole, 2008; Mulcahy et al, 2010, 2014; Gonçalves-de-Albuquerquea, 2015; Jeukens et al., 2017*) and *Enterobacter* (*Rice, 2008*) species. To overcome extended-spectrum beta-lactamase (ESBL)–caused resistance problems (*Pitout, 2008*) carbapenem antibiotics (*Papp-Wallace et al., 2011*) were developed, but carbapenem- resistant (CRE) Enterobacteriaceae (*Temkin et al., 2014*); and *Klebsiella* (Gupta et al., 2011) appeared soon. Lately, the rediscovered and rehabilitated colistin was considered as a final trump (*Kádár et al., 2013*) until colistin resistance was found in Gram-negative bacterium species, (Otter et al., 2017). Antibiotics are also used in plant medicine (*Mc Manus et al., 2002; Stockwell, Sundin and Jones, 2002*; *Aćimović et al., 20*15), but the increasing number of streptomycin-resistant *Erwinia amylovora* isolates has been causing serious problems both in the USA (Förster et al., 2015) and in Europe (Gusberti et al., 2015).

Environmentally-friendly plant, - veterinary, - and human antibiotics of novel modes of action are imperatively needed. Antimicrobial peptides (AMP) have been hoped to provide perspectives. AMPs have found in practically all known prokaryotic a eukaryotic organisms (*Jenssen, 2006*; *Ötvös and Wade, 2014*; *Mojsoska & Jenssen, 2015*). AMPs are mostly of broad target spectra and of strong antibiotic activity. The patented AMPs have recently been listed, (Kosikowska and Lesner, 2016) http://dx.doi.org/10.1080/13543776.2016.1176149, but relatively few of them is in use.

The options of finding novel natural has recently been revolutionized by tools provided by bioinformatics, allowing curation and comparative analysis of genomic and bioinformatics metabolic data of potential antibiotic producing organisms (*Vallenet et al., 2013*); especially since the discovery of the "On-Demand Production" of bioactive natural products, (*Bode et al., 2015*). The symbiotic bacterial partners of the entomopathogenic nematode / bacteria (EPN/EPB) associations (*Steinernema / Xenorhabdus* and *Heterorhabditis / Photorhabdus*) produce antimicrobials (Akhurst, 1982; Forst & Nealson, 1996) mainly AMPs (*Vivas & Goodrich-Blair, 2001); (Bode, 2009*). Their natural role of this antimicrobial compounds is to provide monoxenic

conditions for the respective EPN/ EPB) complex in polyxenic (insect cadaver; soil) environmental conditions. All known EPB-produced AMP compounds are non-ribosomal peptides (NRP), which means that they are synthetized enzymatically by multi-enzyme thiotemplate mechanisms using non-ribosomal peptide synthetases (NRPS), fatty acid synthase (FAS)-related polyketide synthases (PKS), or a hybrid biosynthesis thereof (*Reimer & Bode 2014*). Some examples are xenocoumacins (*Park et al., 2009*); a novel new lysine-rich cyclolipopeptide family (*Gualtieri et al., 2009*) from *Xenorhabdus nematophila*; and the cabanilasin from *X. cabanillasii* (*Houard et al., 2013*).

Our team identified novel some *Xenorhabdus* species (*Lengyel et al., 2005*). Two of them, *X. budapestensis* (EMA) and *X. szentirmaii* (EMC), bacterial symbionts of the nematodes *Steinernema bicornutum* and *S. rarum*, respectively, have been proven exceptionally great antibiotic producers (*Furgani et al., 2008); Böszörményi et al., 2009; Vozik et al., 2015*). The cell-free culture media (CFCM) of *X. budapestensis* (EMA) exerted a strong antimicrobial effects in different bioassays on antibiotic multiresistant laboratory strains, zoonic, veterinary and clinical isolates of *S. aureus, E. coli, Klebsiella*, (*Furgani et al., 2008*); and *Salmonella*, (in preparation); several *Clavibacter, Xanthomonas*, isolates *Phytophthora* species, phytopathogenic *Pseudomonas,* (*Fodor et al., 2012*) as well as in *E. amylovora* rifampicin and kanamycin resistant strains both *in vivo* and *in planta*, (*Vozik et al., 2015*) and on *Leishmania donovani* isolate (*Fodor, Kulkarni & McGwire*, unpublished).

Fellow scientists in other laboratories confirmed our findings. Not only *X. budapestensis*, *(Fuchs et al., 2012)* but also *X. szentirmaii* is a source of antimicrobial compounds of great potential, (Gualtieri et al. 2014). Szentiamide, proved a powerful anti-*Plasmodium* molecule, (Nollmann et al. 2012). A respected French team sequenced our EMC strain (*Gualtieri et al., 2014*). Our data on EMA and EMC (*Lengyel et al., 2005; Furgani et al, 2008; Böszörményi et al, 2009*) were carefully re-evaluated by Bode and his associates. They sequenced EMA, but they did not make it publicly available. They discovered that the most powerful antimicrobial non-ribosomal peptide (NRP) compound is fabclavine, which is present in isomeric forms both in EMA and EMC (*Fuchs et al, 2012*). Comparative genome-analysis identified the hybrid NRPS-PKS gene cluster of 61 kb in both EMA and EMC that is responsible for coding enzyme activities acting in the fabclavine biosynthetic pathway. However, Bicornutin A, erroneously proposed previously as being the active potent antibiotics of EMA by us, does not have any antimicrobial potential (*Fuchs et al., 2012; Fuchs et al., 2014*); although it is usually present in antimicrobially active peptide preparations. The fabclavines are structurally similar to cationic antimicrobial peptides (Lin et al., 2013), which are "displaying pronounced synergistic effects in combination with other antibiotics. This could even increase their bioactivity *in vivo* in combination" (*Fuchs et al., 2014*) with other AMP molecules produced by *X. budapestensis*.

Despite the fact that the most active antimicrobial component (the fabclavine) produced by *X. budapestensis* (EMA) has been discovered and condemned as being generally toxic, the "EMA story" may not necessarily be terminated, since we found resistant organisms in nature the nature; demonstrating that it the EMA AMPs are not an overall ("sulfuric-acid-like") poisons.

Either this is the case or not, the antimicrobially extreme strong EMA-AMP complex seems to be useful model for studying resistance problems related to peptide-type antimicrobial in the nature.

At this point we have become focusing on the resistance / sensitivity problems rather than to the options of the immediate application. Considering that there seems to be more realistic to working on developing the natural complex of interacting natural released by EMA to an agriculturally applicable bio-product (such as compost component) rather than developing on fabclavine with the aim of by disarming this toxic molecule either by chemical or biological (posttranslational mutagenesis, for instance, *Wright et al., 2016*) we worked with a well reproducible peptide isolate, EMA_PF2 and tested in the soil-born plant pathogenic bacterium, *Agrobacterium*. We previously worked on *Agrobacterium* as a plant pathogenic target (*Fodor et al., 2012*) and found *Agrobacterium* strains of different S/R phenotypes to cell-free culture media of EMA and EMC in both overlay and agar-diffusion tests. We decided to try to benefit from the sophisticated genetic toolkit established by fellow researchers on *A. tumefaciens* as number 1 tool of molecular plant biotechnology, (recently reviewed by Nester, 2015).

The aim of this study is to develop an amenable experimental system for studying resistance mechanisms toward natural individual and complex antimicrobial peptides in the future. The advantages of *Agrobacterium* as an experimental genetic system in our study can be summarized as follows:

As well-known, *A. tumefaciens* DNA consists of the indispensable genome DNA (bacterial chromosome, C58 (*Wood et al., 2001); Henkel et al., 2014*); and the dispensable plasmone DNA including a large circular tumor-inducing (Ti) (*Van Larebeke et al., 1974; Currier & Nester, 1976*) plasmid responsible for virulence and tumor-induction in infected plants. The most but not all plasmid-genes are expressed in the bacterium living as vegetative in the rhizosphere. The *vir* genes, which are responsible for virulence, are inducible by chemicals (phenolic, - and sugar compounds) released from wounded plant tissues through the *vir*A membrane histidine kinase receptor. VirA protein then phosphorylates the transcription activator *vir*G, which binds to *vir*-box sequences, located in the promoter regions of *vir* genes (*Koncz*, personal communication).

The genes encoding for enzymes synthetizing of tumor-specific compounds (including opines) are located in the transfer (T-DNA) region that is being inserted into the plant chromosomes, (*Chilton et al., 1977*) and have all signals necessary for expression in plants during crown-gall tumor formation *(Koncz et al; 1983*).

The T-DNA located opine-synthase genes are responsible for the synthesis of respective (nopaline, - octopine, or agropine –type) opines characteristic for a given *Agrobacterium* strain; while enzymes catabolizing (only the respective) opine are located outside of the T-DNA region.

Agrobacterium strains are scored as nopaline (NOP), octopine (OCT) and agropine, as well as L, L,-succinamopine (AGR) opine-catabolizing ones (*Montoya et al., 1977; Guyon et al., 1980*). **(For more details, see Supplementary material (Suppl. Text 1; Table S1).**

A given sensitivity/resistance (S/R) phenotype could be a consequence of more than one mechanism. Genes responsible for S/R phenotypes to EMA_PF2 may be located either on the chromosome; or on the Ti plasmid; or on the second large cryptic plasmid, (in the case of C58 strains, on pAtC58). If S/R phenotypes to EMA_PF2 were plasmid-related, genetic studies could be carried out by complementation analysis in *Agrobacterium*, (Hoekema, 1983). A toolkit for genetic analysis may also include comparisons of S/R phenotypes of strains with different genetic background; such as of different opine type and of plasmid state. For the latter, wild-type, plasmid-cured and helper-plasmid harboring strains producing / catabolizing the same opine are worthwhile to compare. The mutant hunt and mutation analysis of candidate sequences is another way of genetic analysis and reproducible methodology has also been available in *Agrobacterium* (Koekman et al., 1979; Klapwijk & Schilperoort, 1979; Ooms et al., 1980; Ooms et al., 1981; Ooms et al., 1982). There are three more unique attributes provided by the *Agrobacterium* genetic analytical system. First, that the Ti and RI plasmids of different origin are compatible and mutually exchangeable. Second, the "DNA-content" of the T-DNA region flanked by border sequences (Jen & Chilton, 1986) could "freely" be replaced by other sequences. Third, the existence and special function of (prokaryotic) *vir* genes which can mobilize and activate T-DNA cassettes. These genes are coding for *Vir* proteins. The latter play a key role in Type 4 secretion (conjugation of the T-DNA) and processing the T-DNA borders trans by using the *vir*D1/2 relaxation complex , allowing whose function is to mobilize the T-DNA region, (whatever DNA sequences are inside), which cannot be imagine without severely influencing the cell membranes. The greatest advantage from our aspects is that they are capable of acting either from cis or in trans position (Csaba Koncz, personal communication).

Many *Agrobacterium* genomes and plasmids are fully sequenced. Furthermore, the researcher can also benefit from the unique option that partial heterozygotes could be constructed for plasmid segments from resistant and sensitive *Agrobacterium* strains; inserting either by a compatible plasmid from another strain; or selected sequences inserted into a binary vector; and applying complementation strategy called binary vector strategy, suggested by (Hoekema, 1983).

2. MATERIAL AND METHODS

2.1. Bacterium Strains

2.1.1. Source of the strains and culture

EMA and EMC *Xenorhabdus* strains originated from the Fodor Laboratory (*Lengyel et al., 2005*). *Agrobacterium tumefaciens* (HP1836 – HP1843) strains were from the frozen stock collection of F. Olasz. HP1836 - HP1840 had been deposited there by B. Dudás; HP1841 by D. Silhavy; HP1842 by V. Tisza, and HP1843 by G. B. Kiss. *Agrobacterium tumefaciens* SZL1, SZL2, SZL3, SZL4, and SZL5 were provided by László Szabados, (BRC, Hungarian Academy of Sciences, Szeged, Hungary. *Agrobacterium* and *Xenorhabdus* strains were grown and cultured according to the respective routine protocols of (Ausubel et al., 1999); Leclerc & Boemare, 1991); Wise Liu and Binns 2006) All the *in vitro* bioassays were carried out in Luria Bertani broth and/or Luria Bertani Agar.

2.1.2 Antibiotics producing *Xenorhabdus* strains

Xenorhabdus budapestensis (EMA) isolated from *Steinernema bicornutum* was discovered and identified by us (*Lengyel et al., 2005)*. Samples were deposited in DSMZ (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7B, - 38124 Braunschweig – Germany; (http://www.dsmz.de) as DSM16342 in 2004. It has also been deposited in Hungary (asAF13); and also in the Laboratory of Professor Heidi Goodrich-Blair (Department of Bacteriology, University of Wisconsin –Madison, Madison, WI, USA) as HGB033. A spontaneous rifampicin resistant mutant strain was isolated from HGB033 by András Fodor and also deposited there as HGB2238. (Some comparative tests also used the antibiotic-producing *X. szentirmaii* HGB036, as well as the spontaneous rifampicin resistant HGB2239 strain isolated from HGB036 by A. Fodor). All information concerning keeping, culturing, fermenting and bio-assaying EMA has previously been reported (*Furgani et al., 2008; Böszörményi et al., 2009; Vozik et al., 2015*).

2.1.3 Test organisms

2.1.3.1 Test organisms used for evaluating the antimicrobial potentials of peptide-preparations

As a Gram-negative test organism, the double resistant (Km^R; Cm^R) HGB2226 *E. coli* strain was constructed in the Laboratory of Heidi Goodrich-Blair (in the Department of Bacteriology of the University of Wisconsin - Madison, Madison, WI, USA). The plasmid pPG1: Tn10 Km, a derivative of pLOFKm (vector: Ap®, Tn10: Km) had provided by János Kiss, which had been constructed in the Arber Laboratory (Switzerland) by inserting a *Cm* cassette into the *Amp* region. pPG1 was introduced into S17 λ *pir* to make strain HGB2226 by electroporation (by A. Fodor, Kristen Murfin, and Terra Maurer).

As negative (EMA_PF2 sensitive controls, other antibiotic multiresistant resistant ***E. coli*** strains were used, including **HGB 1333 /BW29427** (Dap-requiring, Cm^R) from H. Goodrich-Blair); **ABC 0801** (harboring plasmid with KK88 antigen; KmR, CmR, SmR, TcR); **ABC 1609** (with plasmid TcA1; Km^R, Ap^R, / Sm^S, Sp^S, Gm^S); **ABC 0156** (TG90nal^R; R55 with integrated SG11 genomic island; Cm^R, Km^R, Sul^R, Sm^R, Ap^R, Rif^R, Ery^R) from F. Olasz; **ABC 0785** (hly+, sta, stb; plasmids: pTC, 18ac; Tc^R, from B. Nagy); **ABC 1611** (Serotype: K12; pR16A; Km^R, Ap^R, Sm^R, Sp^S, Gm^S) from P. Dublet, (personal com); **ABC 1499** (Human clinical isolate, Km^R, Gm^R, Cm^R, Flo^R, Sm^R, Tc^R) from F. De la Cruz (personal com.; **ABC 0280** (Human clinical isolate $A3^R$; Cm^R, Km^R, Sul^R, Sm^R, Ap^R, Rif^R, Ery^R) A. Cloeackert, personal com. Also *Salmonella* strains: ***S. tiphymurium* ABC 0159** (Natural isolate, SG11 genomic island, Cm^R, Ap^R Tc^R, Sm^R, Rif^R); ***S. tiphymurium* ABC 0208**, (Natural isolate, SG11 genomic island; Cm^R, Nal^R, Ap^R Sm^R, Tc^R, Rif^R); ***S. enteritidis* ABC 0741,** (Natural isolate, pFOL1111; Ap^R); ***S. enteridis* ABC 1844** (Serotype LT2; recA1; srl-202::Tn10 Tc^R rif^R; Tc^R, Rif^R) and ***S. infantis*** ABC 1748 (Natural isolate Rif^R, Sp^R Ery^R, Su^R sulfamethoxazole / Sm^R) all from F. Olasz.

As a Gram-positive test organism, *Staphylococcus aureus* (SA) JE commercial strain (J.C. Ensign, unpublished) from Dr. J.C. Ensign's Lab was used.

As a fungal target, s the Gram-positive and *Candida albicans* (CA) JE strain (J.C. Ensign, unpublished) was used as a fungal target for testing each preparation for antimicrobial activity in Agar Diffusion Bioassays, which were carried out as described (Vozik et al. 2015)

with minor, actual modifications. This strain was used as an antibiotic double resistant (Km^R; Cm^R) *E. coli* strain.

2.1.3.2. Xenorhabdus strains as test organisms.

HGB1795 is a transposon-induced insertion mutant of the XNC1_2022 gene (Gene ID: 9430524; Gene Page Link: NCBI UniProtKB; Locus Tag: XNC1_2022 see gene page for GenePage for the XNC1_2022 gene EcoGene-RefSeq) from *Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6), provided by Prof. Helge Bode, via Prof. Heidi Goodrich-Blair. The reason why we involved this mutant into this study on EMA_PF resistance studies is that previously Bicornutin A was believed as the active EMA antibiotic molecule, (*Böszörményi et al., 2009* and XNC1_2022 gene of *X. nematophila* was believed to be a homologue of *Xenorhabdus budapestensis Nrp*S (nrpS) gene, (GenBank: Accession Number is JX424818.1; gene synonym="bicA) which is responsible for the biosynthesis of Bicornutin A, (*Fuchs et al., 2012*). It turned out that it is not the case. However, some role in the scenario related to antibiotics activity and self-resistance cannot be ruled out, since the coexistence of Bicornutin A and fabclavine in our peptide-preparations.

Other Xenorhabdus strains were used as positive (resistant) controls, namely *X. budapestensis* HGB033 and HGB2238 (rif^R), *X. szentirmaii* HGB036, HGB2239 (rif^R), *X. nematophila* ATCC 19061 (from S. A. Forst), HGB081 (rif^R), and HGB1789 (rif^R).

2.1.3.3 Agrobacterium strains used in this study

In order to reveal the sensitivity (S) / resistance (R) phenotypes to the antimicrobial peptide complex, we choose *Agrobacterium* strains of different genotype for *in vitro* liquid bio-assaying of EMA_PF2 on them. We worked on strains of different opine type and those on of different plasmid state within the opine groups.

We choose 4 agropine (L, L, - succinamopine, AGR) - catabolizing strains*: A281* (Guyon et al., 1980; Hood et al., 1986); *AGL1* (Lazo, Stein and Ludwig, 1991); *EHA105* (Hood et al., 1993); and *A4T* (White and Nester, 1980; Petit et al., 1982; Jouanin et al, 1986; Slater et al, 2009). All of them are C58Rif^Rstrains. All but A4T have a C58 ("S") chromosome - (the abbreviation indicates the geographic origin (Seattle) of strain A136 (C58 (Rif®), its chromosome also called "Seattle C58"); the sequence of which is slightly different from that of the previously discovered and sequenced "Gent/Leiden C58C" chromosome of nopaline catabolizing plasmid-cured strains (Dr. Paul J.J. Hooykaas, personal communication). **A281** has a wild-type C58 (S) (Rif®) chromosome from one of its ancestor, (the nopaline-catabolizing A136); and an intact, virulent agropine-catabolizing pBo542 [T-DNA] (+) plasmid (from its other ancestor, Bo542). A281 is a hyper, - (Hood et al, 1986; 1987); and also a super, - (Jin et al., 1987)) virulent strain. A known sequence of the pTiBo542 plasmid, outside the T-DNA box (Hood et al, 1986; (Komari, Halperin and Nester, 1986) is responsible for both hyper, - and super-virulence. The intact pTiBo542 plasmid has the T-DNA cassette, containing genes responsible for the synthesis of tumor-opines L, L-SAP, LOP, AGR. The disarmed-DNA deleted remainder sequence, called pEHA 101, contains genes coding for catabolizing enzymes of these opines. **AGL1** is a disarmed derivative of A281 with a mutated C58 (S) (Rif® chromosome with a deletion in the in the *Rec*A gene; its exact genotype is (C58(S), *Rec*A::bla; Rif® Carb®, and is called AGL0; pEHA101. (The pEHA101= pTiBo542 DEL-T-DNA plasmid). The plasmid markers are *Nal*R Mop (+)

(Lazo, Stein and Ludwig, 1991); see also DNA Cloning Service, www.dna-cloning.com). **EHA105** is an indirect derivative of the intact plasmid from A281 (pTiBo542). It was generated from pEHA101 through site-directed deletion of the kanamycin resistance gene on pEHA101 and by Gen® (Hood et al., 1993). (Previously pEHA101 had similarly been created from the wild-type (pTiBo542) when the T-DNA was replaced by KmR, (*Hood et al., 1986*). The genotype is C58(S) Rif® (pTiBo542DT-DNA = pEHA105 / / pBIN-19 – intronGus100-Km®). (See also: (http://www.springerlink.com//content/t02h1486p1862715/). **A4T** is an agropine-catabolizing helper strain of "Gent/Leiden C58C" chromosome; and harbors a T-DNA-deleted (disarmed, helper [T-DNA] (-)] A4T plasmid originated from *A. rhizogenes*; and the binary vector pBIN19 intron (Gus Km®) (*Bevan, 1984*). For more details on *A. rhizogenes* helper plasmid harboring strains and their agro-biotechnological importance, see review (*Taylor et al., 2006*).

As for the NOP strains, we did not have a chance to the virulent wild-type ([T-DNS]) (+) strain. 5 of the 6 (**HP1836** (C58C*-NOP1); **HP1840** (C58C*-NOP2); **HP1843** C58C*-NOP3; **HP1841** (C58C1-NOP4); **HP1842** (C58C1-NOP5) are plasmid-cured, (Uraji, Suzuki and Yoshida 2002); and only the **SZL4** C58C1- pMP90 - NOP6 harbors the disarmed (helper, T-DNA deleted, pTiC58 [T-DNA [(-) called pMP90) plasmid, (Koncz and Shell, 1986). Each of them has C58 chromosome (Wood et al, 2001). The **SZL4** (C58C1-pMP90-NOP6 strain has (the original "Gent/Leiden") C58C chromosome. (Koncz and Schell, 1986), The C58 chromosomes of the other 5 are other (Hungarian) isolate has not been sequenced yet. The genome-selective marker for **HP1836** (C58C*-NOP1); **HP1840** (C58C*-NOP2) and **HP1843** (C58C*-NOP3) strains are naladixic acid resistance (Nal®); while that for **HP1841** (C58C1-NOP4); **HP1842** C58C1-NOP5; and C58C1-pMP90-NOP6 are of rifampicin resistance (Rif®).

As for the OCT strains, we did not have a chance to work either on the ancestor wild-type strain, harboring the virulent pTiAch5 [T-DNA] (+) plasmid; nor on the first disarmed derivative of that plasmid is LBA4213 (*Ooms et al., 1982*); any plasmid-cured OCT strains. We worked on **(HP1837** LBA4404/0-OCT1 and **SZL2** LBA4404/pBIN-OCT2) strains**.** They both have the Ach5 chromosome [38], Henkel et al, 2014), and the chromosomal marker for them is Rif®. Each of the two strains, **HP1837 (LBA4404/0-OCT1)** and **SZL2 (LBA4404/pBIN-OCT2),** strains harbor the disarmed T-DNA deleted helper plasmid pAL 4404; (as known, encoding genes needed for both T-DNA transfer; and octopine degradation (Klapwijk, & Schilperoort, 1979; Dessaux et al., 1988). The plasmid marker is Sm.

All these are summarized in **Table 1** (see Tables).

2.2 Preparation of Antimicrobial Peptide Complexes from EMA

(For more details: see Supplementary Material, Table S2)

2.2.1 Preparation of Sterile Cell-Free Culture Media (CFCM) and Antimicrobial Active Peptide Factions from *X. budapestensis* (EMA) strains

2.2.1.1 Isolation of Amberlite XAD 1148®-bound Methanol-Eluted Peptide-rich Fraction (PF)

The preparation of cell-free conditioned media (CFCM), and obtaining an antimicrobials active peptide-rich fraction (PF) from EMA by using amberlite adsorption followed by methanol (MeOH) elution, as published by ourselves earlier (*Furgani et al., 2008;*

Böszörményi et al., 2009; Vozik et al., 2015). Samples of 10 mg/ml stock solution were kept frozen and then diluted freshly to 1 mg/ml working solutions just before each experiment. New preparations from HGB033 (deposited in UW Madison, Madison, WI, USA) and from the spontaneous Rif® mutant HGB2238 *Rif*® from HGB033 were considered and handled as identical due to the HPLC profile and antimicrobial activity on the same targets.

2.2.1.2 Ultrafiltration of Xenorhabdus PF Preparation

1mg/ml water solutions of samples from EMA_PF preparation were administered to Amicon® Ultra15 to separate PF1 of >10,000 Da and PF2 <10,000 Da. In fact, EMA_PF2 was used as a model antimicrobial peptide complex in *Agrobacterium* experiments.

2.2.1.3 Isolation of Antimicrobial Active Peptide Fraction (EMA_{30}) by Reverse Phase Column Chromatography and AN / TF Elution

The cell-free culture medium of the HGB2238 rifampicin resistant EMA strain was loaded onto a reverse phase column. The protocols described (*Bowen & Ensign, 1998; Bowen & Ensign 2001*), were used and modified, it was necessary, by using the *Sigma protocol*. All buffers and stock solutions for column chromatography were filtered through 0.2-µm-pore-size filters and autoclaved before use. The Sigma protocol was modified by Professor J. Ensign (unpublished) and we used his modified method. Briefly, the column was eluted with a mixture acetonitrile (AN), CH3CN in 0.1% TFA (trifluoroacetic acid) at a flow rate of 0.4 ml/min at room temperature, that is 0, 10, 20, 30, 35 40, 50 and 70 V/V% of AN containing 0.1% TFA. RPCC fractions were named by the number of the concentration of AN, that eluted them from the column. The antimicrobial active peptides from EMA cell-free culture media was quantitatively eluted as one single faction by 30 V/V AN (containing 0.1% TFA) and called EMA_{30}. It exerted strong anti-Gram-positive, anti-fungal and anti-Gram/negative activity (data not shown) and was used for biochemical characterization.

2.2.1.4 Antimicrobial active HPLC fractions

Each HPLC sample was of given volume of a distilled water-dissolved and diluted freeze-dried antimicrobial peptide-complex solution, and, depending upon the column, the respective volumes were loaded, following the protocol. The HPLC protocols we used as described by *Carr* (2002). The eluent absorbance at 218 and 280 nm was routinely monitored. The peaks were detected at 168 -215 nm and 168-280 nm, respectively. Fractions were collected corresponding to the appearing peaks. Both EMAPF2 (the first HPLC sample was called af3; and the second run af6), and EMA_{30} (called AF103) were subjected to HPLC. As for af6, three HPLC peaks were detected, and 5 fractions from below the latest peak (called A2) exerted strong cytotoxic activities on both Gram-positive (SA) and Gram-negative (EC) targets; (see Results). Each experiment was repeated at least twice.

Three peaks from below the main peak of AF103 (called AF103-40; AF103-43 and AF103-44) exerted strong anti-Gram-negative, anti-Gram-positive and anti-*Candida* activity. These fractions were collected on 40^{th}, 43^{rd}, and 44^{th} minutes of the 60-min long HPLC run. None of the other fractions showed anti-Gram negative activity. These fractions were used in MALDI analysis.

2.2. Bioassays of Antimicrobial Peptide Complexes from EMA

2. 2. 1 Methodology of Liquid Bioassay of EMA PF on *Agrobacterium* strains

In vitro Liquid - Culture Bioassays of EMA PF on *Agrobacterium* strains were carried out in sterile 96-well tissue culture plates. Briefly, each culture had 200 µl in the final volume; containing 100 µl of 2X LB (supplemented with the respective selective antibiotics) and 95 µl of a sterile water solution from the partially purified EMA PF, and inoculation of 5 µl bacterial LB suspension from 100 µl; which contained 1 loop-size bacteria from single test bacterium colony grown on LBA plate in 24-h. For the three replicates, 3 independent clones were used. Purified, freeze-dried and re-dissolved preparations of EMA PF were used at 0, 30, 46, 60 and 75 µg/ml concentrations. We incubated the experimental plates for 24h at 28 ^{0}C, and then determined the OD values spectrophotometrically. The growth of bacteria was quantified on the basis of optical densities (OD values) of the cultures by screening the plates spectrophotometrically. The lower OD values indicated the stronger antibacterial activity of the EMA PF and higher sensitivity of the *Agrobacterium* strain tested. Other technical details of the experimental conditions of Liquid Bioassays had been published earlier, (*Fodor et al., 2012; Vozik et al., 2015*).

2. 2. 2. Quantitative evaluations

(If we had worked with a single antimicrobial active compound we should have an exact quantitative parameter if we determined the MIC values. We, however, have had a mixture of peptides of different antimicrobial activity, if we determine the quantitative amount of peptides which exerted a complete inhibitory effect on the tested bacterium strain, this "MIC" values cannot be considered as a quantitative data referring to one active component, but still provide an option for comparing the activity of our EMA_PF2 peptide complex in different strains. Therefore, we determined a value what we named the "gross MIC values" as if EMA_PF2 were a single antibiotic molecule, but we are aware of the fact that it is obviously not the case. The "gross MIC" value is suitable for comparisons of the activities of the EMA_PF2 on different targets, and this is the aim of this study).

Technically the "gross MIC values" of the EMA_PF2 were determined similarly as the MIC value of a single AMP, following the standard protocol, (see References: *Wiegand, Hilpert & Hancock, 2008*; Clinical and Laboratory Standards Institute (*CLSI, 2012*). In fact, we determined the lowest growth-inhibiting dose of EMA_PF2 mixture (and separated fractions) on *Agrobacterium* and control (*E. coli*, *Xenorhabdus, S. aureus*) strains. We used LB broth for dilutions. Briefly, we worked in "SARSTEDT Multiple Well Plate 96-Well Round Bottom with Lid" culture plates, (Sarstedt, Inc., Newton, NC 28658, USA). Test bacteria were inoculated into a liquid growth medium containing different concentrations of EMA_PF2. Growth was determined on the base of the OD values of the liquid cultures, after incubation for 24h (at 28 ^{0}C, when the test targets were *Agrobacterium* and *Xenorhabdus*) and 12h (at 37 ^{0}C, when *E. coli, S. aureus* and *Candida* were the test organisms). When the OD value of a culture did not differ significantly from that of the freshly inoculated LB culture of the same composition, we cautiously considered the applied EMA PF concentration (given in µg/ml) as (gross) MIC90. In case of complete cytotoxicity, we kept the cultures for another two weeks on the bench top and

considered as the final result if no growth was detected during this period of time. The "MIC" /- in fact gross MIC - values added in the Tables and Figures are the means of three replicates. In Agar Diffusion Bioassays we -pipetted 100 µl of samples into a hole in the center of a 1/cm thick LB agar plate. The respective plate was then overlaid by the suspension of the test organism, diluted with soft agar as published earlier (*Vozik et al., 2015*). The diameter of the inactivation zone was measured and the volume of agar media was calculated from that measurement. We considered these data as also informative but preliminary.

2.3 Statistics

The data analysis was performed using [SAS/STAT] software, Version [9.4] of the SAS System for [Windows X 64 Based Systems]; (Copyright © [2013 of copyright]; SAS Institute Inc. SAS, Cary, NC, USA. We used ANOVA and GLM Procedures alternatively following the requirements of the SAS 9.4 Software. The design of the experiment could be considered as a randomized complete block design with the number of the respective treatments, concentrations, and replicates. Data have been averaged as to allow the analysis of variance (ANOVA). The significance of differences of the means (α=0.05) was determined by using t (Least Significant Difference, LSD) tests or Duncan's Multiple Range Tests, depending on the experiment. **(For more details, see Supplementary Material, S_Text_2).**

3. RESULTS

3.1 *Antimicrobial Activity Profile EMA_PF2 and EMA$_{30}$*

Purification, description and of different AMP-preparations made during these experiments are listed in **Appendix Supplementary Material Table S2.** It can be seen that antimicrobial active fractions from EMA_CFCM could be separated either by amberlite adsorption or RFLP, and could be purified by HPLC. Off the preparations which proved antimicrobially active in each of the target organisms we have been dealing those presented in **Table 2** except for EMA_PF1, which was found in very small quantity, and although it was very potent in each target organisms, we could know, whether it contained spontaneously polymerized active peptides, or large, originally inactive peptides which were "contaminated" with smaller antimicrobial active ones. The data of the antimicrobial activity of the different AMP-preparations on Gram-positive (*S. aureus*), Gram-negative (*E. coli*) targets, and on the *X. nematophila* mutant of HGB1795 and *Candida* (fungal) targets determined in Agar diffusion bioassays are presented in **Table 2.**

The data of the antimicrobial activity (measured in two different experiments) of the different concentrations of EMA_PF2 AMP-preparation on HGB1795 mutant and on its two parental *X. nematophila* clones (HGB081 and HGB1789) are presented in **Fig 1A** and **Fig 1B**, in comparison with different negative (*Xenorhabdus*) and positive (*E. coli*) control bacterium strains. The Statistical (ANOVA Procedure) Analysis of the data is present in Supplementary Material.

3.2. HPLC and Maldi Profile of EMA_PF2 and EMA 30

The EMA_PF2 preparation, which was used in the liquid bioassays on *Agrobacterium* strains, could be separated into three sharp peaks after repeated HPLC runs **(Fig 2).** Some but not all fractions collected from below the third (called A2) peak exerted both anti-Gram-positive and anti -Gram-negative activities when tested on *S. aureus* JE and *E. coli* (HGB2226) strains. Anti-Gram-Positive and Anti-Gram-Negative activities could not be separated such a way (**Table 3).**

The fraction collected from below the A2 derived from the HPLC purification of the EMA_PF2 peak exerted strong antimicrobial activity on each tested target strains. The MALDI profile of pooled fractions collected from below the A2 peak (**Fig 3A**) consisted of many peptides. At first sight, there are 5 dense spots within the region of 1300 – 1400 mZ. Many large peaks can be seen in the 1340 – 1366 m/Z and 1373 -1393 m/Z regions, (**Fig 3A**).

The antimicrobially only active RFLP fraction purified from EMA CFCM was $EMA_{30.}$ It was extremely toxic for each of the target organisms. Further purification by HPLC showed that the antimicrobial activity was restricted to peptides collected from the 40 – 57 min of the HPLC run, but only three fractions, collected in the 40^{th}, 43^{rd} and 44^{th} minutes exerted anti-Gram negative activity, on both *E.coli* HGB2226 and *X. nematophila* HGB1795, (**Fig 4).** The MALDI profile of pooled fractions collected from below *AF103_43* **(Fig 3B),** (the most antimicrobial active HPLC fraction on each targets from EMA_{30})**,** consisted of many peptides. On Fig 3B, similarly to Fig 3A, there are large peaks in the 1340 – 1366 m/Z region, but, unlike to Fig 3A, there is no large peak in 1373 -1393 m/Z range (compare **Fig 3B**).

Thus, we figured that peptides between 1340 – 1366 m/Z (believed to involve, 1346 m/Z, fabclavine) were responsible for the antimicrobial activity on four different EMA-sensitive targets.

The MALDI profiles of both the antimicrobial active (**Fig 3 A, B**) and inactive (not shown) HPLC fractions contained many peptide peaks in the range (about 946 m/Z), believed to be where Bicornutin A is located.

3.2 *Results of Liquid Culture Bioassays of EMA_PF2 on Agrobacterium strains of Different Genotype, Opine Type and Plasmid State*

The distribution of OD values as a function of EMA_PF concentrations are presented in **Fig 5.** (As for the respective statistics, see **Supplementary material, Fig S1A – H; Tables S3 & 4**).

Of the 12 tested *Agrobacterium* strains, 8 were resistant to each applied doses (at somewhat different degrees), that is, that is, gross MIC values could not be determined.

One of them was the wild-type AGR strain, HP1838 (A281, of T-DNA (+) genotype).

4 strains were extreme sensitive, (represented by low OD (<0.2) values even at each applied EMA_PF2), which corresponds to the detectable gross MIC values. The common feature of the 4 sensitive strains is that each harbors a T-DNA-deleted (Δ –TDNA) Ti plasmid. 3 of them were of AGR opine type, (A4T, HP1839, SZL3) and one (HP1837) was of OCT opine type (**Fig 5).**

HP1838 was also resistant to unpurified EMA CFCM, while its Δ –TDNA derivative, HP1839 was very sensitive (**Fig 6A** and **B**, respectively). When comparing the OD values of the four AGR strains, it the spectacular difference between the strain (HP1838) of T-DNA (+) and of the three strains (HP1839, A4T and SZL3) of T-DNA (-) genotype. The latter three hardly differed from each other (**Fig A, B, C and D**). (As for the respective statistics related to the results of AGR strains, see **Supplementary material**, **Table S5A, S5B**).

As for the octopine strains, the picture is not so clear. SZL2 is resistant, HP1837 is sensitive, (Supplementary material **Fig S3).**

As for the studied NOP strains, each of them proved resistant to EMA_PF2 in in vitro liquid bioassay. Data on NOP strains are presented in **Supplementary Material, FigS3; Suppl. Text_6; Supplementary Material, S_Text_6; Table S7.** The distribution patterns of the control and that of in the treated cultures are not the same, indicating a moderate and variable cytostatic (but no detectable cytotoxic) effects of EMA_PF2 on the examined strains.

DISCUSSION

We are interested in genetic analysis of natural resistance to natural AMP complexes, such as those produced by EPB nematode.symbibiotic bacteria. We believe that EMA_PF2 is a useful model, independently of its future perspectives as clinical, veterinary, or plant medicine. We did not purify individual peptides and did not determine their contribution to anti-microbial activity of the natural EMA_PF2 AMP complex, since we have been interested in the defense mechanisms against the *natural antimicrobial peptide complex*, EMA_F2, what we chose as a model. Our data indirectly confirm that the predominant component of the EMA_PF2 complex is the fabclavine (Fuchs et al., 2012, 2014), but are interested in the resistance mechanisms of *A. tumefaciens* toward the whole EMA complex of probably interacting antimicrobial peptides. This scenario may better represent the defense mechanisms developed by a soil-born Gram-negative bacterium (*A. tumefaciens*) to the natural antimicrobial peptide arsenal of entomopathogenic nematode bacterium complex.

We do not think that the toolkit of genetics should only be restricted to full-genom analysis, chemical and transposon mutagenesis and physical mapping. But, gene-interactions, such as epistasis, interallelic complementation etc., should be taken into consideration. Consequently, the effective system could provide options for using tools of classical Mendelian genetics. Our goal is to establish an experimental system for genetic analysis of resistance mechanisms against antimicrobial complexes. And, we believe that we found the system we have been looking for. We were not particularly interested in finding resistant mutant to a given AMP molecules, but to find the way to dissect the resistance mechanisms of a species which is resistant to the natural EMA_PF2 natural complex.

The known resistance mechanisms to antibiotics include enzymatic decomposition, efflux pumps (*Nehme and Poole, 2005*), permeability defects, and modifications of target sites (*Fodor et al., 2017*). We suppose that an evolutionarily-built resistance system against a group of interacting

antimicrobial peptides may need another mechanism, the details of which have not yet been discovered. The structural differences of the membranes of cells in the different physiological states, such as presence or absence inducing factors prior to conjugation with the plant cell, may influence the permeability of peptide-like compounds similar to EMA_PF2.

We do not have any inforamtion concerning the membrane structure of cells harboring an intact or a disarmed Ti plasmid. *Christie, (2004*) published that the type IV secretion systems (T4SS) in bacteria are present in *Agrobacterium*, and used to deliver DNA as well as protein substrates from to plant cells. Considering that the T4SS is a complex built up from a several membrane proteins responding to environmental signals, (*Christie, 2004*), this might be a potential clue for understanding the differences between the responses to the studied AGR metabolizing strains.

Considering that the virulent wild-type A281 (HP1838) is resistant we concluded that *A. tumefaciens* is resistant to EMA_PF2. The question is whether this natural resistance has been based on the chromosome, on cross-resistance with other antibiotics, on the Ti-Plasmid, the opine-type, or something else.

Both C58 and Ach5 chromosomes were "represented" among the four sensitive strains as well as amongst the eight resistant strains. This seem to prove that the identity of the chromosome in the S/R phenotype must be ruled out.

Considering that each of the three opine-types was "represented" amongst the 8 resistant strains, the role of the opine type may also be ruled out, although no NOP strain has been found as sensitive to EMA_PF2 in liquid test so far.

The common feature of the 4 sensitive *Agrobacterium strains* strain is that each harbors T-DNA-deleted (Δ –TDNA) Ti plasmid. Three of them are agropine-catabolizing (AGR), and one of them (HP1837) was octopine-catabolizing (OCT). Two of the sensitive AGR strains (AGL1, EHA105) harbor Δ -TDNA pTiA136Bo542, and the third Δ –TDNA A4T (of *A. rhizogenes* origin). Two of them (EHA 105 and A4T) has been harboring a binary vector (pBIN-19-(Intron-Gus-Km®), while AGL1 has not. Each of them was uniformly sensitive (**Fig7B – 7D**). They were also sensitive to EMA CFCM (**Fig 6A**).

At least for the AGR opine group it seems that Δ –TDNA AGR *Agrobacterium* strains are sensitive, while that of intact pTiA136Bo542 plasmid is fully resistant. We do not have information about existence of plasmid-cured agropine strains, so we do not have a chance to determine their S/R phenotype, but our data support the hypothesis that, at least in this opine group is Ti plasmid dependent; more exactly T-DNA dependent.

If the plasmid-cured AGR strains had been viable and sensitive to EMA_PF2, (like the Δ –T-DNA ones), it would have been a proof of the existence of an R-gene, located in the T-DNA region of the pTiA136Bo542 plasmid. If the plasmid-cured AGR strains had been viable and resistant to EMA_PF2, (like the plasmid-cured NOP strains which had been previously reported as resistant to Agrocin 84 as well (Murphy & Roberts, 1979); Ellis, Murphy and Kerr, et al., 1982; Ryder, Tate and Jones, et al., 1984; Farrand et al., 1985; Hayman and Farrand, 1988), it could be interpreted by more way than one.

In the absence of plasmid-cured AGR strains we have only theoretical alternative explanations. Existence of an enzyme converting the non-toxic (or not permeable) EMA-peptide to a toxic (or permeable) molecule which is present (or active) in the sensitive strain but not present (or inactive) in the resistant strains would provide an explanation. The existence of a transmembrane protein transferring the toxic EMA-peptide into the cell which is present (or active) in the sensitive strain but not present (or inactive) in the resistant strains would provide another explanation. Considering that the strains harboring the Ti plasmid but missing the T-DNA cassette are sensitive, while strains missing the Ti plasmid, and consequently the T-DNA cassette are resistant, the only logical explanation is that the permeability (or AMP sensitivity) of the T-DNA deleted Ti plasmid-harboring strains are different from that of both the wild-type and of the plasmid-cured strains. For experimentally testing this hypothesis we need to bioassay EMA_PF2 on T-DNA deleted, plasmid cured and wild type of (T-DNA) $^{+}$ genotype.

Some key experiments, what should be needed to answer some still open question could not be accomplished because of the unavailability of some strains. We did not find plasmid-cured AGR strain available in the literature. Neither we had a chance to bioassay of EMA_PF2 on wild-type ([T-DNA] (+) NOP and OCT strains of intact Ti plasmids. If they happened to be sensitive; like (the wild-type and plasmid-cured NOP strains to Agrocine 84), we have to take the Agrocin 84 model as a more general one. (The resistance/sensitivity of *Agrobacterium* strains to EMA-produced peptides is intriguing and may be based on fortuitous molecular structural similarities (as seemed to be the case for Agrocin 84), even we doubt these two bacterial groups (*Xenorhabdus, Agrobacterium*) would have ever encountered each other in nature). It is more critical that we did not use plasmid- selective antibiotics and cannot exclude the possibility that plasmids from SZL2 and SZL4 might be lost during propagation in liquid culture. Therefore, we should restrict our conclusions to the AGR group of *Agrobacteria.*

We did not have a chance to test wild-type NOP strains, only 5 plasmid-cured stains, each of them proved resistant both to the EMA CFCM (in agar diffusion test) and to the EMA PF_2 (in liquid bioassay); and 1 T-DNA deleted strain (SZL4), carrying a binary vector. Consequently we could not draw any conclusion related to his opine group, as such, even if each studied strain was resistant to EMA_PF2 in liquid bioassay. SZL4 was one of the most resistant to EMA PF_2 (in liquid bioassay, but sensitive to EMA CFCM (in agar diffusion test). There are three theoretical interpretation of this contradicting result. First, that SZL4 was sensitive in a compound present in CFCM but lost during the purification of EMA_PF2. Second: this strain may have lost its Δ-TDNA plasmid during the incubation because we did not use plasmid-selective antibiotics, and therefore behaved, similarly to the resistant plasmid-cured NOP strains. We do not suppose, but could not rule out that presence of the binary vector in SZL4 might explain its resistance to EMA_PF2.

Although to search for S/R phenotypes of *A. rhizogenes* strains was out of the scope of this work, we would like to expand our future research to the strains of this species, because expected similarities and differences between the two species may provide essential information for better understanding the mechanisms of natural resistance developed by these well-characterized

species to multiple antimicrobial peptide complexes, and the joint point is A4T, a T-DNA delated agropine-catabolizing strain of sensitive (S) phenotype to the antimicrobial peptide/complex, EMA_PF2 .

4. CONCUSIONS AND PERSPCTIVES

If we had had only the results on the of L, L,-succinamopine utilizing *Agrobacterium*, strains, we should have been being able to draw unambiguous conclusions from the striking differences between the strong R phenotype of the intact ([T-DNA] (+) pTiBo542 plasmid harboring) A281 and unambiguously S phenotype of all the examined disarmed ([T-DNA] (-) pTiBo542 plasmid harboring) strains, AGL1, EHA 105 and A4T. (Fig 5). These spectacular differences were also proven by the ANOVA-based statistical analyses (**Suppl. material Table S3).**

From the aspects of our goals the possible interpretation of the different S/R phenotypes of different opines types are probably not of primary importance, but we may draw some conclusion

Our data suggest that the EMA_PF2 resistance must be Ti-plasmid related, more exactly T-DNA-dependent.

One of the possible interpretations is that according to the logic of Mendelian basic genetics, - the resistance (dominant) allele(s) should be located in the T-DNA-region of the agropine-catabolizing pTiBo542 plasmid. If it were the case, they must be expressed in our experimental conditions.

The general view is that T-DNA locates genes do not express in the bacterium only in the infected plants. Since the mains stream of the plant biotechnology-motivated research focused on T-DNA genes expressed in the infected plants, the earlier data (of *Schroder et al., 1983*, have almost been forgotten. They discovered (four) protein-coding T-DNA located genes which could be expressed in *E. coli* mini-cells, and their promoter activities were T-DNA located. Their functions have still been unknown, but supposed to mediating growth regulators.

The later discovered pTiBo542 was not subjected to this study, but since then it has been sequenced, and the identified genes from the left border to the right are identified, as follows: agrocinopine synthase; orf_Bo002; IS1312; orf_Bo003; orf_Bo004; orf_Bo005; indole-3-acetamide hydrolase; tryptophan 2-monooxygenase; adenylate iso-pentenyl-transferase; hormone sensitivity modifying enzyme; protein 6b; succinamopine synthases; see https://www.ncbi.nlm.nih.gov/nuccore/DQ058764.1).

Of the predicted products of the unknown open reading frames (they should be: an *A. rhizogenes* rolB/C product-like; a DNA-binding helix; an integrase domain; an IS3-transposase; and an unknown protein, respectively) none of them one should be considered as “resistant gene” at the first sight. On the other hand, there are arguments supporting the hypothesis that (at least in the agropine group) the EMA_PF2 resistance is *somehow* T-DNA related. Each of A281, AGL1 and EHA105 strains have the same chromosome (C58 Rif®) and harboring L, L,-succinamopine utilizing pBo542 strains of the same origin. There are two genetic differences

between the A281 and AGL1 strains, but the question whether any of them could be an explanation of the differences in their S/R phenotype: (1) The presence of the TDNA region of the pTiBo542 in A281 (*Van Larebeke et al., 1974*), and its absence in AGL1; (2) The RecA chromosomal gene in A281 is the wild-type, while in AGL1 it has a deletion (*Lazo et al. 1991*). A281 is completely resistant; while the D-T-DNA AGL1 is fully sensitive to EMA_PF2.

Both AGL1 and EHA105 (*Hood et al., 1993*) harbors of D-T-DNA disarmed plasmid are fully sensitive to EMA_PF2. One of them (EHA105) is *Rec* A (+), and the other (AGL1) is *Rec* A (-). Consequently, the differences in the sensitivities of A281 and AGL1 to EMA_PF2 could not be a consequence of their differences in the *Rec* A locus; but rather of the presence / absences of the Bo542 [T-DNA] cassette. Another supporting argument is that agropine-catabolizing supers-ensitive AGR strain, A4T, (*Petit et al., 1982*, *Jouanin et al., 1986*) also harbors a disarmed, cryptic (RI) agropine-plasmid, not Bo542 but another one from another species, *Agrobacterium rhizogenes*, (*Slater et al., 2009*, see references: *White and Nester,1980*; *Chilton et al., 1982; White et al., 1985; (Endoh et al.1990; Taylor et al., 2006; Mankin et al., 2007*). The only common feature between A4, AGL1, and EHA105 is the absence of the T-DNA cassette from the respective AGR Ti plasmid.

Agrobacterium strains harbouring del-T-DNA plasmids and complementing DNA sequences (either in another plasmid or in binary vectors) within the cells may provide a suitable system for genetic analysis of resistance to antimicrobial peptides other than EMA_ PF2.

Such an experimental system can be based on the existence of super-sensitive del-T-DNA pTiBo542 harboring L, L,-succinilopine-catabolising *Agrobacterium* strains and the beneficial situation that Ti plasmids fully compatible with each other and partial heterozygotes could be produced. The binary vector strategy of Hoekema (1983) could be adopted in such a way.

Despite of our primary interest to study the resistance mechanisms of Agrobacteria to complex interacting anti-microbial peptides, such as those comprise our model, EMA_PF2, it would be especially interesting to screen the previously identified individual antimicrobial peptides of Xenorhabdus and Photorhabdus origin, (see Parke et al., 2009; Gualtieri et al., 2009; Fuchs et al., 2012; Nollman, 2012; Fuchs et al., 2013; Li, Plésiat and Nikaido 2015; Xi et al., 2012); and especially the kolossin A, (Bode et al, 2016) in this *Agrobacterium* system.

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Competing interests

Authors have declared that no competing interests exist.

Authors' Contributions

András Fodor, **Ferenc Olasz** and **János Kiss** designed the study. The experiments were 80% carried out about at Department of Bacteriology, of the University of Wisconsin-Madison, in Madison, WI, USA by Fulbright Research Grantee **A. Fodor**; by doing the bacterial studies in the Laboratory of *Heidi Goodrich-Blair*; Fermentation, HPLC, MALDI analysis and some of the bioassays in the Laboratory *Jerald C. Ensign*. Experiments (prior to Madison) were conducted in A. Fodor's laboratory at the University of Pannonia, Keszthely, Hungary, where EMA_PF were routinely produced by Dávid **Vozik** (**tutor:** Katalin Bélafi-**Bakó);** some bioassays on *Agrobacterium* strains were carried out by Ahmed Nour **El-Deen** and others by Erzsébet **Böszörményi**. Other experiments (just before and right after Madison) were carried out at the University of Veterinary Medicine, Budapest, Hungary by A. **Fodor**, László **Makrai** and László **Fodor.** Ferenc **Olasz,** János **Kiss and** László **Szabados** and provided *Agrobacterium* strains with the proper guidance, while Steven A. **Forst** provided the *X. nematophila* strain ***var-2***. The statistical analysis was carried out by **A. Fodor** with the guidance of Muhammad Akbar Bin Abdul **Ghaffar** at the Ohio State University in Wooster, OH. J. **Kiss,** F. **Olasz** A. **Fodor** with the professional proofreading of Michael G**. Klein** made the manuscript completed. The final shaping of the figures was performed by **J**. **Kiss.**

Data availability

The following information was supplied regarding to data availability:

Experimental Data Analyzed by ANOVA Procedure: provided in the Supplemental Files

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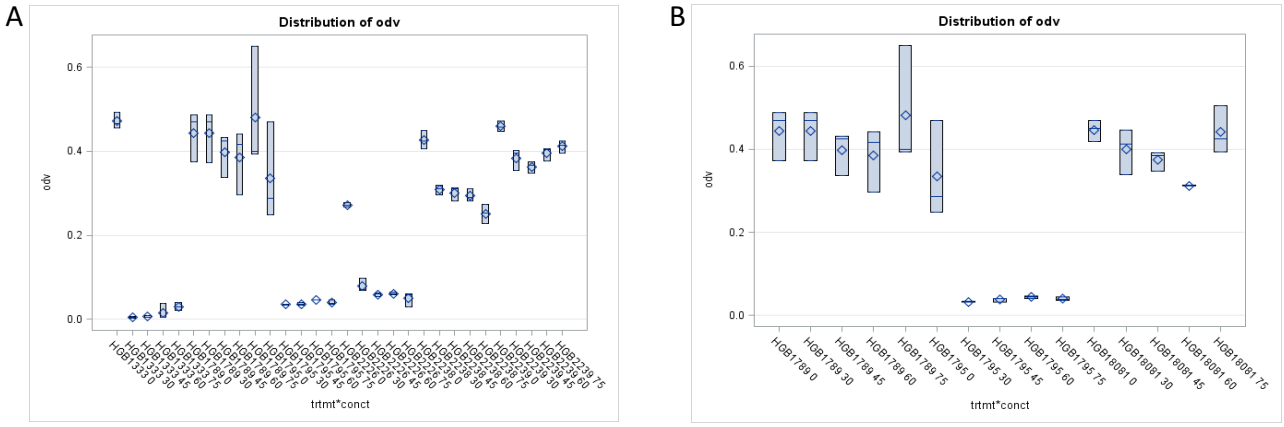
Figure 1(on next page)

Effects of EMA_PF2 on Xenorhabdus end E. coli strains: OD values determined in in vitro liquid bioassays

X. nematophila HGB1795 is extremely sensitive to EMA_PF2. (A) Comparison of the OD values of HGB1795 and positive (E.coli HGB2226 and HGB1333) and positive (*X. budapestensis* HGB2838 and *X. szentirmaii* HGB1839) control strains treated with different doses of EMA_PF2. (B) Comparison of the OD values of HGB1795 with those of its parental strains deposited in Madison (USA) and Germany. For more details, see Caption to Fig 1.

Antimicrobial Activity of EMA_PF2 on *E. coli* and *Xenorhabdus* strains. Results of Liquid Culture Bioassays



In **Fig 1A** the distribution of the OD values (Means +/- StDev) measured in 24-h cultures of *Escherichia coli* (strains HGB1333 and HGB2226, grown at 37 °C) and those of *Xenorhabdus budapestensis* (HG2338), *X. szentirmaii* (HGB2339) and *X. nematophila* HGB1789 wild type strains (grown at 28 °C) demonstrate the strong anti-Gram-Negative activity of the antimicrobial peptide fraction EMA_PF2 isolated from the cell-free-culture of *X. budapestensis* (HGB033) against which not only the cells of the EMA-producing species, *X. budapestensis*, but those the wild-type strains of *X. szentirmaii* and *X. nematophila* cells are completely protected. (Not all data are given, but each tested *X. nematophila* strains, including *X. nematophila* Types Strain ATTC 19061 deposited in Madison as well, as HGB800; and *var-1* (Völgyi, Fodor and Forst, 2002); all tested *lrp* mutants characteristically resembling to secondary form strains (Cowles et al., 2007) were unambiguously resistant to EMA_PF2, Fodor et al., unpublished). Strain HGB1795, however, which had been isolated as a Tn insertion mutant from the spontaneous rifampicin resistant *X. nematophila* strain HGB081 (of H. Goodrich – Blair, a spontaneous mutant a derivative of *X. nematophila* Types Strain ATTC 19061, see Materials and Methods) proved as sensitive as the examined *E. coli* strains. To confirm this interesting discovery, we repeated the test in an independent experiment, in which HGB1795 was treated with another preparation of EMA_PF2, in comparison with the original HGB081 deposited in the laboratory of Prof. H. Goodrich-Blair and that clone of this mutant from which H. Bode isolated a transposon-induced insertion mutant of the XNC1_2022 gene what was then deposited in the Laboratory of H. Goodrich-Blair in Madison, WI as HGB1795, see data on **Fig 1 B**.

Abbreviations: Abscissa, trmt*conc: 0, 30, 45, 60 and 75 µg/ml EMA_PF2; 10081 = *X. nematophila* spontaneous rifampicin resistant parental strain HGB081; 1789 = *X. nematophila* strain HGB1789, a clone of HGB081; the parental strain of HGB1795; HGB1795= transposon-induced insertion mutant of the XNC1_2022 gene isolated from HGB1789 (HGB081); HGB 1333 and HGB2226 = *E. coli* (negative control) strains; HGB2238 and HGB2239: spontaneous rifampicin-resistant (positive control) strains isolated from *Xenorhabdus budapestensis* HGB033 and *X. szentirmaii* HGB036 by A. Fodor; *odv* = optical density of the respective bacterium culture

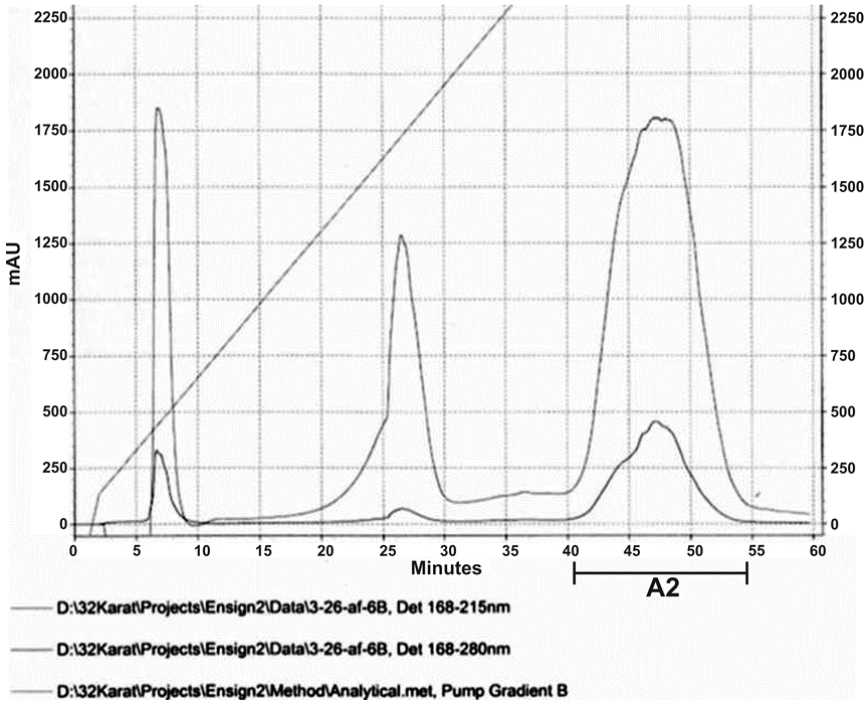
These (Fulbright-sponsored) experiments were carried out in the Laboratory of Professor **Heidi Goodrich-Blair using** her equipment (spectrophotometers and other things) and bacterium strains, at Department of Bacteriology, University of Madison, Linden Drive, Madison WI, and the USA.

Figure 2(on next page)

HPLC profile of EMA_PF2

Off the fractions collected from below the three large peaks only those exerted detectable (strong) antimicrobial activities which were collected from below A2. For more details, see Caption to Fig 2.

High Pressure Liquid Chromatogram (HPLC) Profile EMA_PF2.



EMA_PF2 is peptide- fraction **(EMA_PF2),** which had been isolated from cell-free culture media (CFCM) of *X. budapestensis* (EMA) by Amberlite XAD1148® adsorption; purified followed by gradient MeOH elution; and eluted by with 99V/V% of methanol, followed by sterile ultrafiltration (*Vozik et al., 2015)*. HPLC peaks were detected at 168 -215 nm and 168-280nm, respectively. When tested in Agar Diffusion Bioassays Fractions collected from below the third (A2) peak (between 40-45 min intervals) exerted strong antimicrobial activities on both Gram-positive (*Staphylococcus aureus,* strain JE) and Gram-negative *(Escherichia coli* strain HGB2226) test organisms, (see Table 2) and also on *Candida albicans* (not shown). Fractions collected from below the peaks at the first B2, (5-10 min), and the second, B1. (18-30 min) intervals showed no antimicrobial activity on either target. This experiment was carried out in the Laboratory of Professor Emeritus **Jerald C. Ensign** using his equipment, solutions and standard methods, Department of Bacteriology, University of Madison, Linden Drive, Madison WI, and the USA. Maldi analysis of the most active fraction pooled samples from below the A2 peak three peaks are presented in Fig 3A.

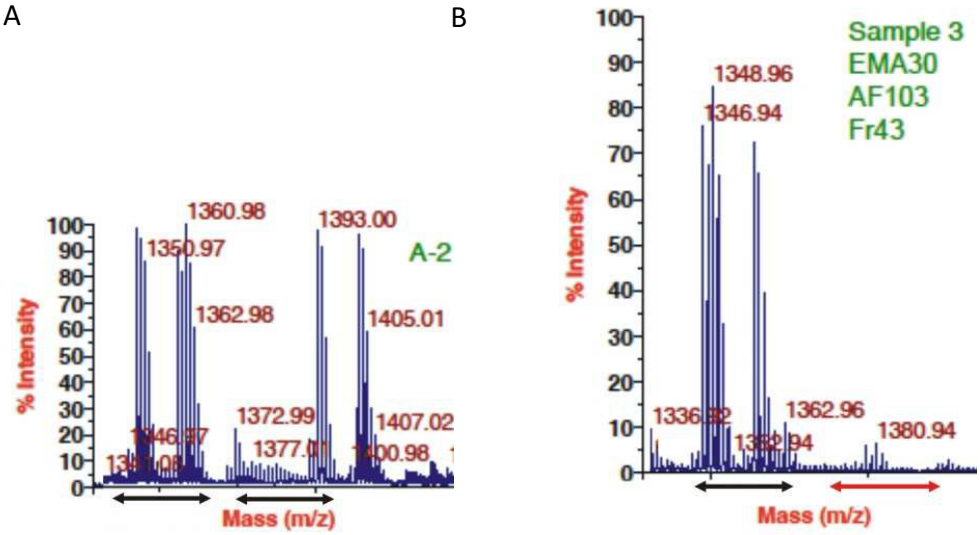
Figure 3(on next page)

MALDI profiles of two antimicrobial peptide complexes obtained from EMA cell-free culture media by different protocols.

(A) MALDI profile of pooled antimicrobial fractions from below A2 peak of the HPLC profile of EMA_PF2. (B) MALDI profile of EMA 30, purified by RPLC. For more details, see Caption to Fig 3.

Comparison of the MALDI profiles of two antimicrobial active preparations from the cell-free culture medium (CFCM) of *Xenorhabdus budapestensis* (EMA).

A B



The complete MALDI profile of the pooled fractions from below A2 peak of the HPLC chromatogram (Fig 2) of the Amberlite-Adsorbed, MeOH-purified EMA_PF2 at three magnifications (not shown)

Each peptide-peak is < 2,200 m/Z. There are many peaks in m/Z range, where Bicornutin A is expected to be present (500-1000) and the there are many in the m/Z range of 1,200-1500, (lowermost chromatogram); but the majority are located between 1,300-1,400 (uppermost diagram). **Fig 3A** shows this region in more details. Two arrows indicate the most densely populated sub-regions.

The HPLC profile of purified peptide preparation obtained by column chromatography (EMA30, (AF103), is very similar (not shown). The MALDI profile of the most active antimicrobial HPLC fraction, Fr43 of EMA30 (AF103) is rather similar (**Fig 3B**) but not identical to that of A2.

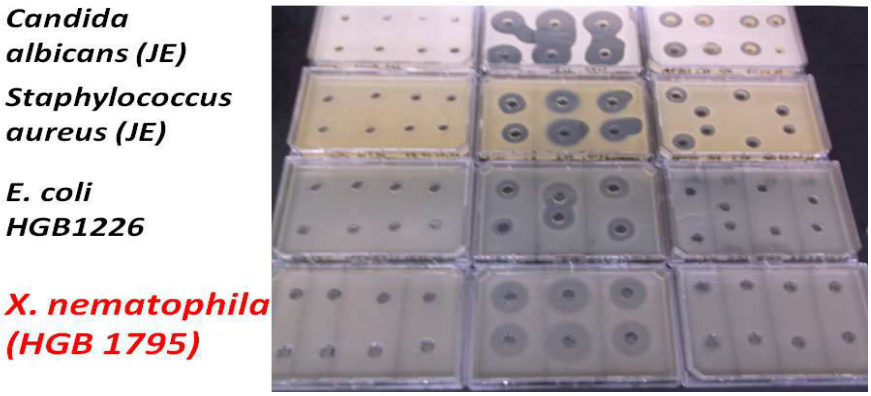
The main difference is that only the left sub-region of the m/Z range on Fig 3C has peptide peaks, the right one, labelled with red arrow is almost empty. The left molecular-region is where fabclavine isomers (*Fuchs et al., 2012, 2014*) are expected to be located, and this region is very similar on Fig 3B and 3C. The m/Z regions were Bicornutin A is expected to be localized is very similar in 3B and 3C. This observation may be considered as an indirect confirmation that the predominant active antimicrobial peptide component of EMA AMP complexes is the fabclavine, but Bicornutin A (or similar peptides in other *Xenorhabdus* species, with of unknown role) may also be present in antimicrobial active bio-preparations.

Figure 4(on next page)

Comparison of the antimicrobial activity of peptide fractions separated from HPLC from EMA(30) on 4 different targets in Agar Diffusion Bioassays

Test organisms: SA = *S. aureus* JE; as a Gram-positive; EC= E. coli HGB2226; as a Gram-negative target; CA= *Candida albicans* JE, as fungal target; HGB1795: an insertion mutant of *X. nematophila* with extreme sensitivity to *Xenorhabdus* antimicrobials. Columns of holes: the places into which 0.1 ml of samples were pipetted. from left to right: sample collected in the 19th, 21st, 23rd, 25th, 40th, 43rd, 44th, 45th, 49th and 57th min of HPLC run. For more details, see Caption to Fig 4.

Growth inhibiting activities of AF_103 (EMA30) HPLC fractions on different targets in Agar-Diffusion tests



Each hole had been filled with 100 µl volume of a sample, overlaid with the uniformly soft-agar diluted cell-suspension of the respective test organism. Samples (holes) from left to right **Left plates**: fractions collected at retention time 19th, 21st , 23rd and 25th from below the first peak of HPLC run (Fig. 1), **Plates in the middle**: fractions collected at the 40th, 43rd and the 44th minutes retention times; from below the largest sharp peak of HPLC run, **Right plates**: fractions collected at the 45th, 47th, 53rd and 57th minutes from below the last large, not-very-sharp peak (at the end of the chromatogram). **From top to bottom**: Sensitive test organisms: CA =*Candida albicans*, SA = *Staphylococcus aureus,* EC =*Escherichia coli* HGB2226, *X. nematophila* HGB 1795, two replicates of each.

Table 1(on next page)

Agrobacterium strains used in this study

Genotype: includes **genome that** is the respective chromosome (C58; Ach5) with genome-selective markers; and the **Plasmon** (including the **Ti**(**RI**) plasmid with plasmid/selective markers and the respective **binary vector**(**BIN)** with binary vector selective markers). The binary vectors are engineered DNA constructions which are capable to replicate in both *Agrobacterium* and *E. coli* cells and could be transmitted to plant cell as well because they include T-DNA border sequences which could be recognized by the respective *vir* gene product. ***Abbreviations of the selective markers*** : **Nal®, Rif®; Carb®; Ge®,** and**Sm®** : resistant to naladixic acid, rifampicin, carbenicillin, gentamycin, and streptomycin, respectively. **T-DNA**: a special segment (cassette) of the Ti (RI) plasmid which could covalently be inserted into the infected plant chromosome. It carries genes expressed and regulated in the infected plant cell.

T-DNA$^{(+)}$: genotype of the wild-type Ti plasmid having the intact T-DNA cassette;**(Δ –T-DNA) means: [T-DNA]$^{(-)}$** genotype of the disarmed (non-virulent, helper) Ti (RI) plasmid from which the T-DNA cassette had been precisely excised. **Opine:** opines are strain-specific compounds, synthesized by the respective opine-synthase gene in the plant tumors and can also be catabolized by decomposing enzymes encoded in the respective Ti (RI) plasmid, located outside of the T-DNA cassette For more details: see Captive to Table 1).

Table 1: *Agrobacterium tumefaciens* strains used in this study

Agrobacterium strain							
Name		Genotype					
LAB	REF	Genome		Plasmon		T-DNA	Opine
		Chromosome	Selective marker	Ti plasmid	BIN		
HP1836	C58C*NOP1	C58C*	Nal^R	Cured		$[T\text{-}DNA]^{(-)}$	NOP
HP1840	C58C*NOP2	C58C*	Nal^R	Cured		$[T\text{-}DNA]^{(-)}$	NOP
HP1843	C58C*NOP3	C58C*	Nal^R	Cured		$[T\text{-}DNA]^{(-)}$	NOP
HP1841	C58C1NOP4	C58C1*	Rif^R	Cured		$[T\text{-}DNA]^{(-)}$	NOP
HP1842	C58C1NOP5	C58C1*	Rif^R	Cured		$[T\text{-}DNA]^{(-)}$	NOP
SZL4	C58C1/ pMP90NOP6	C58C1*	Rif^R	pMP90 Ge^R	pHP9-Gus101	Δ– [T-DNA]	NOP
HP1837	LBA4404/0 OCT1	Ach5	Rif^R	pAl4404 Sm^R		Δ– [T-DNA]	OCT
SZL2	LBA 4404/ pBIN-OCT2	Ach5	Rif^R	pAl4404 Sm^R	pBIN	Δ– [T-DNA]	OCT
HP1838	A281	C58	Rif^R	pTiA136 Bo542		$[T\text{-}DNA]^{(+)}$	AGR
HP1839	AGL1	C58C* (AG0)	Ca^R $RecA^{(-)}$	pEHA101Nal^R		Δ– [T-DNA]	AGR
SZL1	EHA 105	C58C*	Rif^R	pEHA105Nal^R		Δ– [T-DNA]	AGR
SZL3	A4T		Rif^R	A4T		Δ– [T-DNA]	AGR

Captive / Footnotes to Table1 *Agrobacterium tumefaciens* strains used in this study.

Genotype: includes **genome that** is the respective chromosome (C58; Ach5) with genome-selective markers; and the **Plasmon** (including the respective **Ti** (**RI**) plasmid with plasmid/selective markers and the respective **binary vector** (**BIN)** with binary vector selective markers). The binary vectors are engineered DNA constructions which are capable to replicate in both *Agrobacterium* and *E. coli* cells and could be transmitted to plant cell as well because they include T-DNA border sequences which could be recognized by the respective *vir* gene product.

Abbreviations of the selective markers: **Nal®, Rif®; Carb®; Ge®,** and **Sm®**: resistant to naladixic acid, rifampicin, carbenicillin, gentamycin, and streptomycin, respectively.

T-DNA: a special segment (cassette) of the Ti (RI) plasmid which could covalently be inserted into the infected plant chromosome. It carries genes expressed and regulated in the infected plant cell. **T-DNA $^{(+)}$:** genotype of the wild-type Ti plasmid having the intact T-DNA cassette; **(Δ –T-DNA) means: [T-DNA] $^{(-)}$** genotype of the disarmed (non-virulent, helper) Ti (RI) plasmid from which the T-DNA cassette had been precisely excised. **Opine:** opines are strain-specific compounds, synthesized by the respective opine-synthase gene in the plant tumors and can also be catabolized by decomposing enzymes encoded in the respective Ti (RI) plasmid, located outside of the T-DNA cassette.

Abbreviations of opines: NOP =nopaline catabolizing (and synthetizing) *Agrobacterium* strain; OCT = octopine catabolizing (and synthetizing) *Agrobacterium* strain; AGR=agropine and L, L, - succinamopine catabolizing (and synthetizing) *Agrobacterium* strain.

Abbreviations of strains from which the complete Ti (RI) plasmid had been removed: **The C58 strains cured for the pTiC58 plasmid general C58C* means C58 cured. The C58 strains cured especially for the pTiC58 (rifR) Ti plasmid are labeled as C58C1.** C58C1 means that the cured stain carries a chromosomal rifampicin resistance mutation. C58C1RifR was alternatively designated as GV3101. **C58C*** = (in this study) the cured stain carries a chromosomal naladixic acid resistance mutation. We did not have a chance to work on plasmid-cured OCT and AGR strains.

For details on plasmids (pMP90; pAl4404; pTiA136Bo542; pEHA101; pEHA105 and A4T) see text and **Supplementary material TextS1.**

Table 2(on next page)

Agar-diffusion bio-assays of antimicrobial active peptide fractions from EMA_CFCM on four sensitives targets

EMA_PF1, EMA_PF2, (both purified by Amberlite adsorption) and EMA 30, obtained by RPLC) proved extreme active. Both EMA_PF2 and EMA30 could be further be purified by HPLC, but EMA_PF2 lost its anti-Gram(+) and Gram(-) activities during further purification by RPLC. For more details, see the captive.

Table 2 Agar-Diffusion Bioassays of Antimicrobial Activities of Peptide Fractions Isolated from EMA CFCM: Inactivation Zone Sizes (IZ values) Determined in Four Targets Agar-Diffusion test.

Antimicrobial active peptide preparation	Inactivation zone in mm^2			
	Gram negative targets			
	HGB2226		**HGB1795**	
	N	Mean ± SD	N	Mean +/- SD
EMA_PF1	3	3820.00±690.22	3	4280.00 ±415.69
EMA_PF2	4	3683.75±799.23	2	6602.50 456.08
EMA_PF2*20		0.00	3	3370.00± 635.83
EMA_PF2*30		0.00		3119.00 ±842.61
EMA_PF2*40		0.00		4088.00± 678.70
EMA_PF2*50		0.00		3821.67 ±214.20
EMA_PF2*70		0.00		4640.00± 850.97
EMA_PF2*TF		0.00		4172.00±1502.02
EMA_$(RPLC)_{30}$	3	1452.50 ±95.45	3	1761.33± 173.78
AF103_(EMA)_HPLC40	3	617.00±88.02	3	1135.33± 119.52
AF103_(EMA)_HPLC43	3	1614.00± 81.41	3	2073.33 ±244.32
AF103_(EMA)_HPLC44	3	1019.33 ±113.52	3	1385.00± 100.00
	SA		**CA**	
	N	Mean ± SD	N	Mean ± SD
EMA_PF1	3	8723.33 ±600.44	3	11746.67 ±704.37
EMA_PF2	3	5931.67± 453.22	3	6291.6667±627.58134
EMA_PF2*20		0.00	1	530.00 ±0.00
EMA_PF2*30		0.00	3	696.33± 279.69
EMA_PF2*40		0.00	3	544.67 ±226.68
EMA_PF2*50		0.00		0.00
EMA_PF2*70		0.00	3	623.33 ±175.65
EMA_PF2*TF		0.00		558.33± 49.07
EMA_$(RPLC)_{30}$	3	1656.67± 40.41	3	1526.00±233.83
AF103_(EMA)_HPLC40	3	1614.00 ±81.41	3	2289.00±0.000
AF103_(EMA)_HPLC43	3	1886.33± 66.97	3	2930.00± 287.51
AF103_(EMA)_HPLC44	3	1613.67± 81.98	3	2834.33± 377.57

Captive / Footnotes to Table 2: Agar-Diffusion Bioassays of Antimicrobial Activities of Peptide Fractions Isolated from EMA CFCM: Inactivation Zone Sizes (IZ values) Determined in Four Targets Agar-Diffusion test.

Antimicrobial Activities of Peptide Fractions Isolated from EMA CFCM were determined in four targets in Agar-Diffusion test. Inactivation zones were calculated from the diameter of inactivation zone in 1-cm tick LBA plates and given in mm^3.

Abbreviations:

AMP Fractions:

EMA_PF1: A peptide fraction isolated from the cell-free culture medium (CFCM) of *Xenorhabdus budapestensis* (EMA). EMA_PF1is a fraction supposed to be containing molecules of MW > 10,000 D. We suppose that the large peptides of PF1 this fraction adsorbed the smaller active peptides and we detected the activity of the complex rather than antimicrobial activity of peptides of MW > 10,000 D.

EMA_PF2 (PF2) consists of peptides of MW < 10,000 D;
EMA_PF2*20, EMA_PF2*30, EMA_PF2*40, EMA_PF2*50 and EMA_PF2*70: EMA_PF2 was further fractionated by**, RPLC,** and fractions eluted by 20. 30, 40, 50 and 70 V/V% of AN containing 0.1% TFA, freeze-dried, taken up by PBS and bio-assayed. **TF:** fraction which did not adsorb to the column.

EMA_ (RPLC) 30 or (EMA30) also mentioned as **AF103** (as HPLC sample) is a purified fraction of EMA PF by Reverse Phase Column Chromatography; eluted with 30 V/V% AN. (This was the only RPLC fraction active on Gram-negative targets).
AF103_ (EMA) _HPLC40, AF103_ (EMA) **_HPLC43** and AF103_ (EMA) **_HPLC44** HPLC sub-fractions of EMA30 (AF103) collected in the 40th, 43rd, and 44th minute of the run of sample AF103.

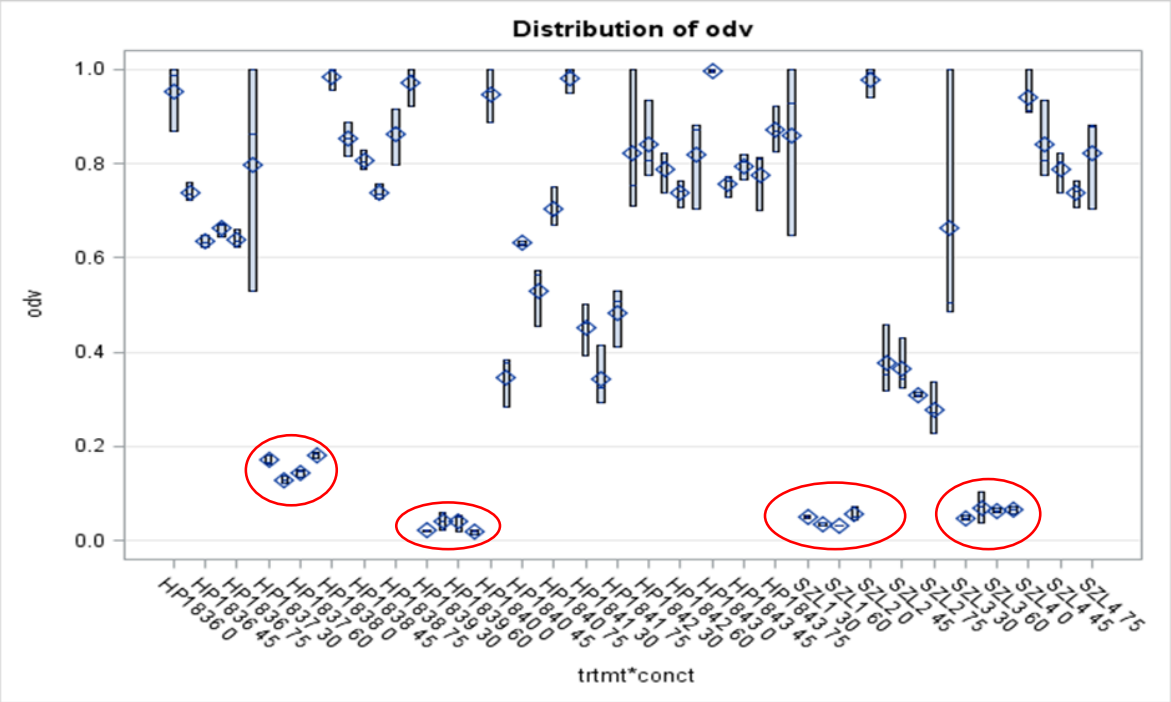
Test organisms: HGB2226 = *Escherichia coli* (*E. coli*) mutator strain of Km® Cm® genotype, constructed by us (as a Gram-negative target); HGB1795 = a transposon-induced insertion mutant of the XNC1_2022 gene (Gene ID: 9430524; Gene Page Link: NCBI UniProtKB; Locus Tag: XNC1_2022 see gene page for GenePage for the *XNC1_2022* gene EcoGene-RefSeq) from *Xenorhabdus nematophila* (strain ATCC 19061 / DSM 3370 / LMG 1036 / NCIB 9965 / AN6), kindly provided by Prof. Helge Bode, via Prof. Heidi Goodrich-Blair, which behaves like an immune-suppressed *X. nematophila*, a *Xenorhabdus*-antibiotic-sensitive *Xenorhabdus* mutant; SA = *Staphylococcus aureus* (as a Gram-positive), CA = *Candida albicans* as fungal target, respectively.

Figure 5(on next page)

Distribution of the OD values measured in in vitro liquid bioassays of EMA_PF2 in 12 *Agrobacterium* strains

Note that (the gross) MIC values could be determined only for 4 *Agrobacterium* strains, HP1837, H1839, A4T and EHA105, not for the others. Each of the sensitive ones is Δ –TDNA strain. All the others should be considered as resistant ones but in different degree. For more details, see Captive o Fig 5. For raw data see Supplement.

Summary of the results of the *in vitro* liquid bioassays of EMA_PF2 in Agrobacterium strains of different genotype, opine type and Ti plasmid state.



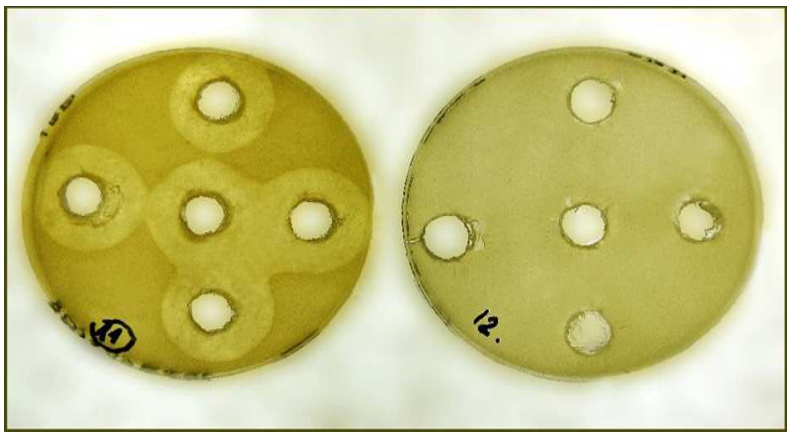
The mean values and standard deviations of OD *(odv*) were obtained from 3 replicates. Ι istribution of the OD values measured in 24h liquid cultures of 0, 30, 45, 60 and 75 µg/ml dose of EMA_PF2. The *odv* of each strain (HP1836; HP1837; HP1838; HP1839; HP1840; HP1841; HP1842; HP1843; SZL1; SZL2; SZL3; SZL4) were determined at each concentration. Because of space limit, not all data are noted on the abscissa. Data were analyzed by ANOVA procedure. **Abbreviations**: Odv = values of optical density (OD) determined spectroscopically, trmt*conct: ANOVA results of the strain (treatment) and dose (concentration) interaction (*); which is hardly different from those of the freshly inoculated LB culture controls. These four data groups of OD values are of those of the 30 and 60 µg/ml dose of EMA PF treated HP1837; HP1839; SZL1 and SZL3 cultures, respectively. These *Agrobacterium* strains are considered to be of S phenotype (sensitive to EMA PF), while the rest of the strains are R phenotype (resistant to EM A PF), even if they are not uniformly resistant**.**

Figure 6(on next page)

Comparison of the sensitivities of the wild-type, virulent (A281) and a T-DNA deleted (Δ –TDNA) derivative (EHA105), detected in Agar Diffusion Bioassay. Both of them are L, L,-succinamopine, AGR) - catabolizing Agrobacterium strains.

A: EHA105; B: A281. Both of them are L, L,-succinamopine, AGR) - catabolizing Agrobacterium strains. Inactivation zones around the holes in the LB-Agar plates, containing 0.1 ml volume of samples of EMA_PF2.

Agardfiffusion bioassay of sterile cell-free culture medium (CFCM) of nematode-symbiotic bacterium *Xenorhabdus budapesensis* (EMA) on agropine—catabolizing (L,L,-succinamoipine utilizing, AGR) *Agrobacterium tumefaciens* strains.



Left: EHA105, a T-DNA deleted (Δ –T-DNA) strain. **Right**: A281, the virulent, wild-type (T-DNA (+) strain. **Plasmid genotypes**: EHA 105 (pTiBo542 (T-DNA(-) = (pEHA105); Nal^R Mop(+)). A281: (+) pTiBo542 (T-DNA(+) HP1838 (A281, right)) *Agrobacterium* strains. (Photo: Ahmed Nour El-Deen)

Table 3(on next page)

Anti-Gram-positive and anti-Gram-negative fractions peptide-fractions obtained from EMA_PF2 by HPLC.

The antimicrobial active fractions could be collected between the 40 - 50 min period of HPLC run. The Gram-negative and Gram-positive fractions could not be separated. For more details, see the Caption

Table 3 Anti-Gram Positive and Anti-Gram-Negative Activities of HPLC (EMA_PF2) isolated from the Cell-Free Culture Medium (CFCM) of *Xenorhabdus budapestensis* (EMA) by Amberlite XAD1148® Adsorption, Methanol Elution, and Ultrafiltration in liquid culture bioassay

	OD Values at 600 nm for the fractions collected between 40-50 min (Mean ± SD, n=2 at each time)	
HPLC run	***Staphylococcus aureus* JE**	***Escherichia coli* HGB2226**
1.	0.3577±0.0797	0.5380± 0.009
2.	0.4404±0.0511	0.4214±0.0002
3.	0.4273±0.0377	0.4335±0.0002
4.	0.4588±0.0307	0.4625±0.001
5.	0.4027±0.0285	0.48135±0.00063
6.	0.3874±0.0510	0.4651±0.00198
7.	0.4255±0.0571	0.4395±0.0004
8.	0.0003±0.0247	0.00155000±0.0006
9.	0.0003±0.0201	0.00020000±0.0002
10.	0.0081±0.0547	0.001±0.0001
11.	0.0040±0.0061	0.0015±0.0002

Captive / Footnotes to Table 3: Table 3 Anti-Gram Positive and Anti-Gram-Negative Activities of HPLC **(**EMA_PF2) isolated from the Cell-Free Culture Medium (CFCM) of *Xenorhabdus budapestensis* (EMA) by Amberlite XAD1148® Adsorption, Methanol Elution, and Ultrafiltration in liquid culture bioassay

Anti-Gram Positive and Anti-Gram-Negative activities of HPLC Fractions from EMA_PF2

Abbreviations:

HPLC Sample: af3; Peak: A2

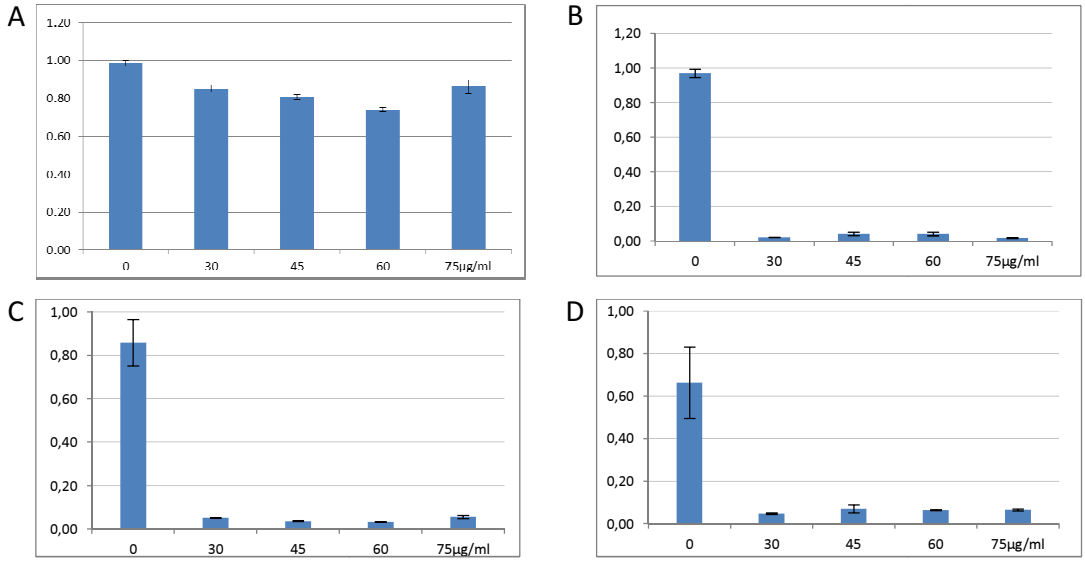
Fractions collected (from below A2, between 40 – 47 min exerted complete cytotoxic activities on both the Gram-positive and the Gram-negative test organisms. Fractions collected before and after this time-interval were completely inactive. Each mean was calculated from 3 replicates. In repeated experiments, using in different columns got similar results. (We were not interested in the growth rates of the bacterial cultures; only in the completely toxic fractions. This explains the layaways from the original protocol).

Figure 7 (on next page)

OD values measured at different concentration of EMA_PF2 in liquid bioassays in the L, L,-succinamopine, AGR) catabolizing *Agrobacterium* strains

OD values (mean +/- SE; N=3) measured in 0, 30, 45, 60 and 75 microgram/ml of EMA_PF2 in strain (A) A281 (HP1838); (B) AGL1 (HP1839); (C) EHA 105; (D) A4T. For genotypes and more details, see Captive to Fig 7).

Results of *in vitro* liquid bioassays of EMA_PF2 agropine—catabolizing (AGR) *Agrobacterium tumefaciens* strains.



A: on wild-type A281 (HP1838) *Genotype*: C58 pTiA136Bo542 – TDNA (+) Rif^{R}; **B**: AGL1 (HP1839). *Genotype*: C58 (AG0) (RecA::bla) pTiA136Bo542 – TDNA (-) Rif® Ca® Nal® Mop (+). **C**: Fig 7C EHA105 (SZL1). *Genotype*: C58 pTiA136Bo542 DTDNA (-) / pBIN-19-(Intron)-Gus-Km® Rif® Nal® Mop (+) Km®; **D**:A4TC (SZL3). *Genotype*: C58 A4TC/pBIN-19 – (Intron-Gus-Km(R) Abscissa: EMA_PF2 dose µg /ml. Ordinate: OD values.