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Project acronym **ET-PA**
Project title **Enabling Techniques for the Development of a novel Class of Protein Antibiotics**
Instrument **Horizontal Research Activities involving SME - Co-operative Research Project**

Thematic Priority **Cell Penetration Peptides, Antibiotic Resistance, Novel Class of Antibiotics;**

ET-PA Final Report

Part 1: Final Activity Report

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Project coordinator organisation name **ADRIACELL S.R.L.**
Revision **[final]**

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ET-PA Program - General Considerations

The quality of medical care has widely benefited from the introduction of antibiotic agents, both anti-infective and anti-neoplastic, which have been central to the increase in life expectancy and our quality of life. A serious and growing problem with their extensive use, however, is the growing rate of drug resistance phenomena; a problem compounded by the fact that the development of new drugs is lagging. We risk losing the benefits gained from these drugs and any new class of antibiotic agent that can contribute to novel therapeutic options is a valid pursuit.

"Targeted destruction of the genetic programs by REPP technology alone or in synergism with approved drugs can provide us with a new concept to combat the evolving of resistant mutations and erase adaptation with a single strike."

(Mission statement, Christian Kuehne, ET-PA coordinator)

Enabling Techniques for the Development of a Novel Class of Protein Antibiotics (ET-PA - www.et-pa.org)

Executive Summary

Resistance of pathogens to antibiotics is a major threat to public health and safety, increasing the risk of mortality, especially in hospital settings. The increased aging of the population in the developed world means that an ever greater proportion develops infections and neoplasias of different types. Currently the market for antibiotic agents is dominated by small molecule classes, which are all facing increased drug resistance and requiring product differentiation. While this remains a primary focus in anti-infectives and anti-tumour development, new concepts for entirely new classes of substances opening novel therapeutic options are essential.

Project objectives:

The ET-PA project was focused on the implementation of a generic platform to enable the development of a new class of protein-antibiotics; rationally modified, single-chain class II restriction enzymes (RE) fused to cell penetration peptide sequences (PP). These REPP compounds allow selective target cell penetration and inactivation. The power of the REPP principle is that it lends itself equally to the development of potential antimicrobial agents and powerful anti-neoplastic leads; the selective factor is the specific PP.

The key technology behind this platform is based on inventions proprietary to the participating SMEs that enabled maximal freedom to operate during the entire ET-PA program: (i) REPP principles and their application by Adriacell; (ii) isolation and purification via the novel CIM chromatographic techniques provided by BIASEP.

Background

RE's are homodimeric enzymes produced by bacteria to defend themselves against infection by bacterial viruses (phages), that cut bacterial DNA at specific sites. Bacteria that produce a particular RE are protected against it, but others that don't produce it would suffer extensive damage to their DNA, so that REs can act as effective protein antibiotics. However, this requires engineering the enzymes so that they can penetrate into susceptible pathogens, and be present in an active form even at low concentrations. This is why the ET-

PA project intends fusing an appropriate cell penetration peptide sequence (PP) to an engineered restriction enzyme (RE) that includes both subunits in a single chain, so as to produce a "REPP" construct capable of microbial cell penetration and autonomous folding to an active unit within the cell (**Figure 1**).

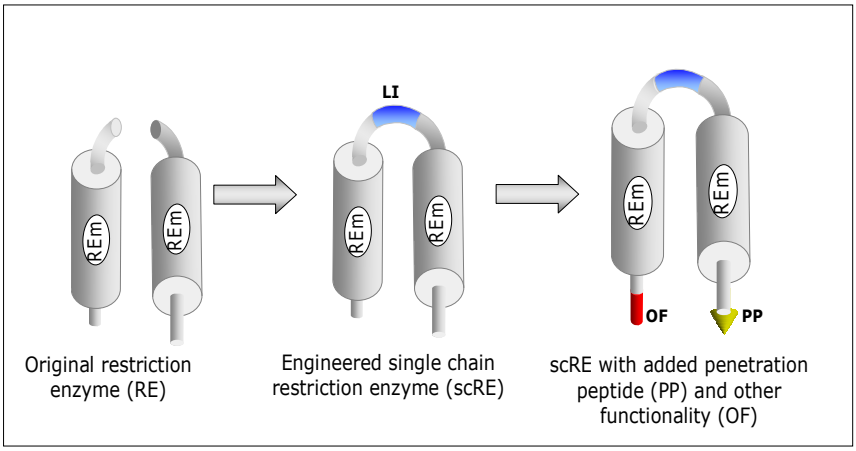

Figure 1: ***Design process and REPP construction.*** The original restriction enzyme, PVUII is a homo dimeric molecule (REm). This protein has been redesigned to be a single chain by insertion of an appropriate linker region (LI) between the two parts. A penetration peptide sequence (PP) can then be added to one terminus and another functionality (OF) to the other terminus, to assist translocation into bacteria or increase activity.

Penetration peptides (PP) have been extensively studied for targeting biopharmaceuticals to eukaryotic cells, but less is known about penetration into bacterial cells. One of the principal aims of the ET-PA project is to identify such peptides. Excellent candidates include the antimicrobial peptides produced by the host-defence systems of animals and plants. Many of these are membrane active, acting either by damaging the bacterial membrane or translocating into bacteria to reach internal targets. By fusing these peptides to an engineered single-chain RE, they can carry it into cells as "cargo", allowing it to reach the bacterial DNA (**Figure 2**). This is a denaturing process, so the enzyme cannot pass as a dimer. The advantage of a single-chain REPP is that re-folding to the active enzyme within the cell occurs in single molecules even at very low concentrations.

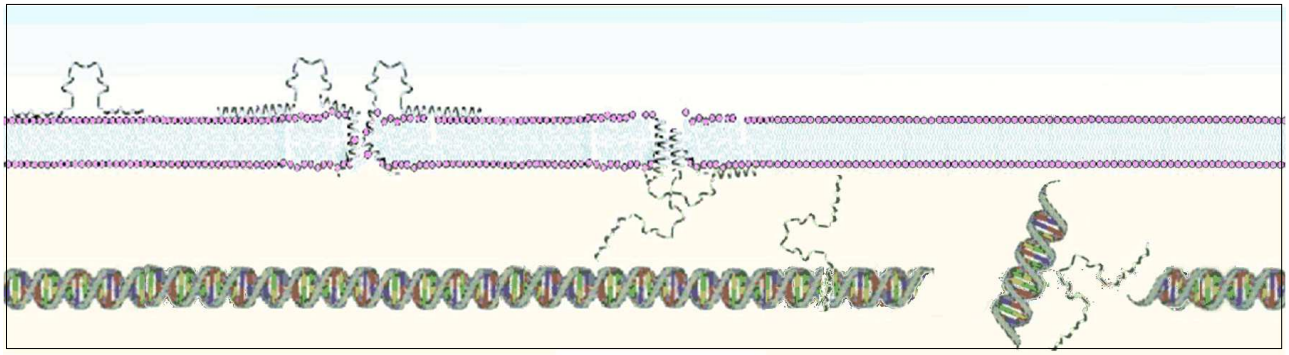

Figure 2: ***Proposed mode of action of antibiotic REPPs.*** The engineered proteins are attracted to the anionic bacterial surface as they are cationic. The penetration peptide (PP) sequence then aids internalization by pore formation or assisted translocation. This process may require collaboration between several REPP molecules. Once inside the bacterial cell, the REPP molecules efficiently bind to and cleave its DNA, effectively inactivating the pathogen.

The ET-PA concept thus uses a natural system that has evolved as the primary prokaryotic defence shield against horizontal DNA transfer, originally discovered for bacteriophages. Horizontal DNA transfer is also one of the main routes by which antibiotic resistance is selected. Moreover, some of the naturally occurring colicin proteins, produced by some bacteria to defend their ecological niches from rival strains, show similar features for an albeit narrower host range (e.g. E2, E7, E8, E9 colicins display DNAse activities as their action mechanism). The fact that these relatively large proteins internalize provides a strong support for the ET-PA concept. REPP constructs are expected to constitute highly efficient antibiotics by killing bacterial cells through DNA hydrolysis, and have the potential for a direct targeting of drug resistance.

Outcome for Project Partners

The ET-PA project brought together three SMEs (ADRIAC, BIASEP and HECUS) and two RTD performers (UTS-BC and UBIMMI) from four different European countries, which are leaders in their field. An outcome of the project was the creation and testing of a solid R&D network that will continue to collaborate in the future. The primary scientific and technological outcome was the successful development of a technology platform that allows for the construction and screening of large REPP protein libraries. This platform will be applied for further lead development, not only with respect to antibacterial REPPs, but also with respect to the third generation of analogous REPP compounds under an ongoing tumor-therapeutic developments program in one of the SMEs, ADRIAC. In constructing the REPP platform that was the central ET-PA objective, all SME partners gained profitable value added to their businesses. REPP platform development also brought about consolidated prototypes for CIM Monolytic purification device applications in high throughput (HT) genomics and proteomics, benefiting the producer Biaseparations, as well as Adriacell. The interdisciplinary nature of the project also created the critical mass for entry into the medical applications market for the third SME in designing thermo-elements and software for the existing Small Angle X-ray scattering device pallet of the company and allows them now to leverage their share in the growing proteomics and genomics R&D market. The RTD partners contributed design features, helped characterize and validate REPP constructs and CIM devices and conducted assay development and screening.

Dissemination and Application of Results

REPP technology is now strongly supported by the successful A venture round of the coordinating SME, ADRIAC, whose success depended in significant measure on the activities of the ETPA project. The successful application of CIM technology during the project allows BIASEP to access the biotech market, and along these lines the potential HT applications can also be considered a successful outcome of ET-PA. The third SME, Hecus, has grown by acquisition of a complementary company during the ET-PA period and has now the critical mass and in house expertise to further integrate instrumentation developed during ET-PA outcome into their product portfoglio. RTD partners have instead been able to develop methods and find exploitable outcomes for their ongoing studies, contributing also to dissemination of some results via publications in international journals and technical handbooks.

Work Performed

The ET-PA consortium had defined that for a successful targeting of cells by REPP antibiotics these molecules must accomplish ***four tasks***:

- cross the outer cell wall barriers and bind to the membrane surface;
- enter into the cytoplasm by translocating in a receptor dependent or independent manner;
- fold to the active form - a process whose rate limiting step in natural REs is dimerisation;
- specifically bind and cut genomic DNA;

Task 1) During the project, data was collected that indicated that for efficient crossing of the outer cell wall layers the REPPs molecules needed to overcome potential stability constraints, structural and size constraints, as well as constraints deriving from unspecific interactions with extracellular matrixes that would sequester the REPP molecules, and that depended significantly on the nature of the PP portion. Standard antimicrobial activity assays were found to be insufficient for an adequate characterization of REPP antibiotic activity and were re-evaluated. New types of assays were also considered. Strategic masking of the REPP molecules with compatible homopolymers was perfected and successfully applied. REPP stability issues were also definitively resolved. REPP constructs with promising antimicrobial activity have been identified, but most important, REPP design is now consolidated and has become more predictable. The advantages and limits of this platform are now clearly defined. Furthermore, the platform can be extended to various applications beyond antimicrobials, such as anti-tumor therapeutics, and this is an enormous added value.

Task 2) Research concerning the targeted engineering of REPPs for membrane recognition, translocation and cell entry was carried out throughout the project. REPP candidates were evaluated for differential pathogen-target specificity and concomitantly for a low toxicity profile for eukaryotic host cells (see **Figure 2**.). While the ET-PA prime objective was delivery of REPP molecules to bacterial cells, a parallel project for targeting tumor cells greatly benefited from the REPP optimization process, in turn providing useful input into ETPA. In fact, in these parallel lines of research the targeting of prokaryotic cells proved to be the more complex for various reasons: i) active concentrations were not as low as expected; ii) assay reproducibility was strongly dependent on conditions and bacterial growth phase; iii) pharmacokinetics proved complex. A main objective in the second period of the project was thus to better understand the bacterial penetration process so as to increase its efficiency. REPP molecules designed for use with eukaryotic cells showed at least three orders of magnitude increase in potency and selectivity during the optimisation process, applying similar strategies. These differences were likely due to the presence of receptor-based import systems in host cells, and in contrast to eukaryotes our understanding of bacterial import systems proved insufficient. In order to proceed to a more rational REPP design for bacteria it was evident that both a better understanding of the entry mechanism, and also more sensitive and robust assay methods were required. For this reason, the RTD partners concentrated on research programs to characterize internalization mechanisms and also to provide alternative and more sensitive assaying.

Task 3 and 4) In the first stages of the project, both single and double chain REPP versions were evaluated and the single chain version was proven to be superior towards a functional folding process. This was perfected and used exclusively during later stages of the project. The single-chain architecture was fully optimized by i) defining the optimal length and amino-acid constraints of the linker region connecting the two RE monomers; ii) improving monotypic folding so as to increase activity; iii) altering the sequence and context of the import sequence to increase overall compound stability; iv) modifying the REPP surface with non-proteic homopolymers to increase compound stability and bioavailability. As many different PP sequences have been analyzed, the general concepts for the PP portion in the REPP molecules could be accurately defined. Understanding the structural constraints (determining folding and CPP presentation) allowed a significant improvement in the design of the REPP molecules themselves. REPP production processes have also been optimized, and REPP solubility and refolding issues critical for the future lead selection process defined.

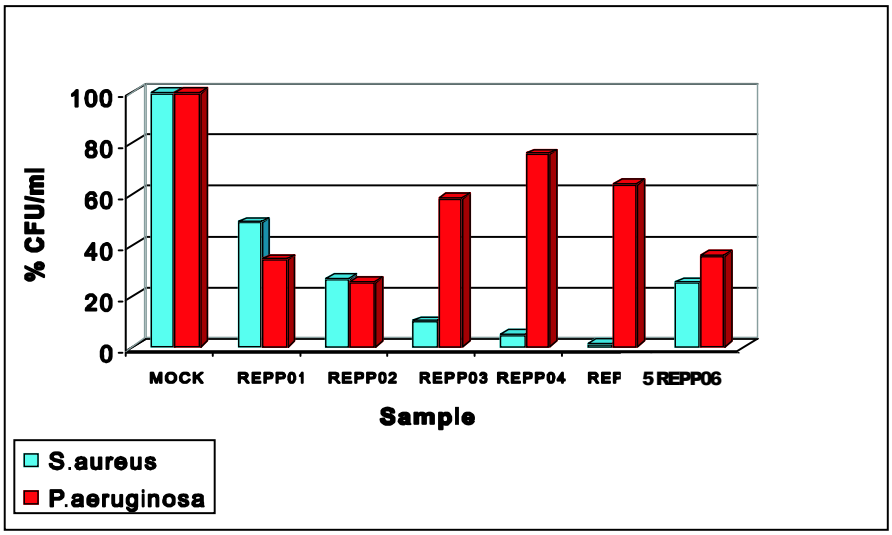

Figure 3A: ***Target specificity screen of PP sequences***. REPP molecules containing different potential PP sequences were tested for target specificity by a bacterial killing assay. Colony forming efficiencies of pathogens was assayed after a single-dose treatment [*Pseudomonas aeruginosa* (Gram negative) or *Staphylococcus aureus* (Gram positive]

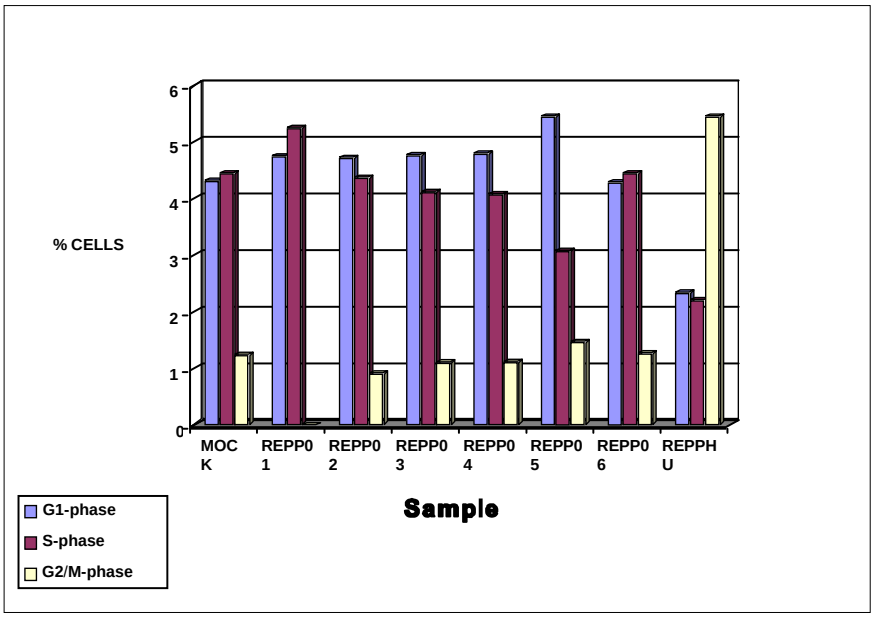

Figure 3B: Toxicity assays for REPP molecules in human cells. REPP molecules containing different PP sequences were tested for toxicity in a Human Osteoblastoma Cell assay developed during the ET-PA project. Results for antibacterial REPPs were compared to a REPP built with a human cell specific PP (REPPHU). Changes in cell cycle profiles after a single dose treatment were assayed by fluorescent activated cell-sorting (FACS) and expressed as a measure of % cells with a specific DNA content. MOCK represents buffer untreated cycling cells and estimates the cell cycle distribution of a proliferating cell-population.

Section 1 – Project objectives and mayor achievements during the project

Overview of project objectives

i) Design, construction and expression of REPP molecules (WP1);

ii) Evaluation of REPP molecules against indicator bacterial strains and identification of active configurations (WP2);

iii) Development of sensitive and robust assays and characterization of CPP internalization mechanism into bacteria (WP2);

iv) Semi-automated production systems and process throughput monitoring systems to allow flexible REPP library production (WP3)

The above objectives were sequential, but interdependent. In the first period, the project concentrated mainly on area i) and ii), which provide the basis for iv). The know-how obtained and problems encountered suggested the need in the second period to develop more sensitive activity assays and to obtain a more in depth understanding of internalization mechanisms (iii). Gains in protein design technologies in the second period allowed to revise the multiplex objectives for area iv) in more detail and now provides for its implementation.

Objectives for the project by workpackage

WP1 - In the initial phases of the first period (P1) of the ET-PA project main focus was REPP design, construction and expression by WP1. Prime goal was to provide REPP prototypes for evaluation early on. This not only brought about first REPP candidates but clearly defined the basic technical strategy for ET-PA from experimental data. Reliability and robustness of used methods were consolidated and early problems arising not only were solved but helped to strengthen consortium relationships and interactions. Progress in the design of REPP constructs was made continuously throughout the project as described in the following individual actions:

i) a detailed evaluation of possible penetration peptides (PP) suitable for internalization of cargo into bacteria, an area not extensively covered in the literature.

ii) conversion of the suggested sequences to suitable oligonucleotide sequences for fusion within an artificial monomeric or single chain RE gene, a process requiring re-evaluation of how to most efficiently construct these genes.

iii) transfection of the constructed genes into a suitable producer bacterial strain, requiring selection of a strain being both immune towards the RE action and suitable for REPP polypeptide expression.

iv) folding of the produced REPP polypeptide to its active form, requiring re-evaluation of the REPP construct itself to increase folding efficiency.

v) determining the stability of REPP molecules to degradation by bacterial proteases, tested by exposing them to supernatants from bacterial cultures for extended periods.

vi) improving the design of REPPs to increase production, stabilise folding to a more active final form, and increase stability of the REPP fusion proteins, in particular regarding the import sequences;

vii) addressing the issue of toxicity and stability by masking the REPP constructs with non-proteic homopolymers;

viii) perfecting the semi-automated production and testing platforms.

These activities were carried out principally by the coordinating partner Adriacell, with aid from the RTD partners (particularly action i) and other SME partners (particularly action viii).

WP2 - In the initial stages of the project, WP2 carried out an evaluation of REPP activity *in vitro*, using standard MIC, growth kinetics and killing assays. Following a critical evaluation of the antimicrobial activity of REPPs and progress made during the mid-term meeting, in the second period the RTD partners of the consortium concentrated on improving the sensitivity of antimicrobial assays and in defining modes of bacterial penetration for future REPPs. Individual actions were:

i) setting up REPP specific bacterial growth inhibition assays, bacterial killing kinetics assays and minimal inhibitory concentration assays,

ii) defining most suitable assay conditions (medium, CFUs exposed, REPP starting concentrations, assay duration etc.);

iii) assessing the activity of different REPP families;

iv) re-evaluating penetration peptides (PP) suitable for internalization of cargo into bacteria, and identification of possible active import mechanisms into bacteria

v) re-evaluating of bacterial targets for REPPs.

vi) developing more sensitive and robust assays suitable for higher throughput screening

vii) assisting the redesign of REPPs with novel PP sequences.

WP3 - Activities were carried out throughout the project concerned with the establishment of the REPP production systems, including automation of production and purification procedures for oligonucleotide building blocks for synthetic REPP construction, and REPP protein constructs. Individual actions were:

i) perfecting a robotic platform for production and/or assaying REPPs

ii) perfecting chromatographic separation processes (monolithic CIM disc protocols suitable for oligonucleotides, based in IEX chromatography and for polypeptides, based on RP chromatography)

iii) establishing structure verification and quality control by analytical HPLC / ES-MS analysis;

iv) developing a 96 CIM disc devices.

v) developing a process control programming flow chart;

vi) defining the principles for a purification device suitable for 96 well CIM-Disk protocols

WP4 - Activities were carried out concerning project management, project coordination and exploitation and dissemination of results, and included:

i) establishing and maintaining the ET-PA project web-site (www.et.pa.org), for dissemination of the project and to stimulate communication within partners and affiliates;

ii) preparing and arranging first contacts towards fund rising from public or private partners for further development of the REPP and CIM technologies for intracellular applications and for finalizing respective topic ET-PA REPP constructs in an IND application;

iii) exploring applications to create an added value for the contained platform technology;

iv) contacting for a third party valuation within the REPP project;

v) filing for patenting of the new stability and PP principles invented in part during the ET-PA program by Adriacell;

Outcomes of the project

a) Producer strains were optimised to increase production levels up to tenfold. The REPP structure was further optimised by redesigning the linker region to improve monotypic folding and increase DNA-binding/cleavage (*activity improvement*), by working on the primary structure and structural context of the PP sequences so as to decrease proteolysis (*intrinsic stability improvement*), and by covalently modifying some surface residues in the REPP construct so as to increase serum stability (*extrinsic stability improvement*).

b) The CIM disk based separation strategies for REPP oligo and fusion protein purification were found to be entirely satisfactory and were integrated in to a preparative chromatographic station for up-scaling.

c) The original strategy based on a custom built process and control software was substituted more cost-effectively with one based on commercial software for DNA construction, which became available during the project. This software was integrated it into a commercially available robotic station. The process control programming flow-chart was defined.

d) Membranolytic and other types of PP sequences were evaluated. An active transport system for Proline-rich PP sequences was identified by one RTD partner into Gram-negative bacteria for use with REPP.

e) More sensitive assays were evaluated based on either luminescent or fluorescent bacterial reporter strains.

f) Proof of principle was achieved for the REPP antibiotic concept and the REPP technological platform.

Main achievements of the project

These are defined by the projected Deliverables, most of which have been completed with little adjustments and delays. Mayor gains are in brief:

✓	Improved understanding of how REPPs work	(**knowledge**) ;
✓	Ability to design, construct and produce REPP prototypes	(**predictability**);
✓	Capacity to redesign REPP constructs for increased activity/stability	(**optimization**);
✓	Improved understanding on how to test REPP proteins	(**read out**);
✓	Optimised REPP production	(**reproducibility**);
✓	96 CIM disk and device as new product for sales	(**productivity**);
✓	Semiautomatic scale REPP production/testing	(**productivity**);
✓	A round venture evaluation of the REPP concept	(**financing**);

An added value to these achievements was consolidation of the network of SME and RTD partners capable of collaborating in an open and friendly manner, which will continue after the end of the project, to the benefit of both sets of partners.

Main problems and corrective actions undertaken

Problems and Corrective actions: Given the highly innovative nature of the ET-PA project, it was expected that it would present demanding, often unexpected and sometimes underestimated hurdles; problems that however were solved overall so that the ET-PA project remained on schedule. These consisted in:

Problem 1) differential solubility, stability and toxicity in the expression systems depending entirely on the PP portion. We learned that selected PP sequences can change REPP biophysical properties strongly; The polypeptide representing the RE active principle of the REPP proteins are highly soluble, but addition of the PP portion, even if less than 5% of the total mass, can markedly influence solubility, folding and toxicity. A single amino-acid change in the PP portion can be decisive. Our experiments suggest that this is because PP sequences render the otherwise hydrophilic REPPs into more amphipathic molecules. The different overall effects observed could be attributed to the antimicrobial peptide structural family from which the PP was derived.

corrective actions. Optimisation was carried out class-wise - PP sequences can be grouped into a limited number of classes and the conditions for only one member then apply also for the rest of the group. This is fundamental knowledge for future multi-well REPP production; The knowledge to produce REPPs with effectively any PP sequence with recombinant techniques has been gained.

REPP class	PP type	Effect observed	Corrective actions
Class I	CPP	solubility decreased	inclusion body refolding
Class II	Leu/Arg/Lys-rich	highly soluble but unstable	Arg → Lys increases stability
Class III	Trp-rich	solubility decreased	inclusion body refolding

Problem 2) Given the intrinsic variability of REPP production, semiautomatic implementation of the procedure seemed complex and our projected linear models to install semiautomatic REPP production had to be consolidated. For this the mayor goal was to develop a simple and unique protocol. This is the main reason why some issues concerning program development in WP3 lagged behind schedule;

corrective actions. Techniques were developed to prepare and test all proteins under denaturing conditions, with all required steps done in a fully automatic mode. At this point, positive candidates from the semiautomatic screen can be optimized for expression (using the class concept described above). The availability 96 well CIM separation technology was central for the automatic REPP production according to this scheme.

Problem 3. Initially, artificial REPP gene construction was by assembly of short oligo-nucleotides into entire genes. The high lethality of the restriction enzyme products however resulted in much higher error-rates during assembly, as compared to neutral gene assembly with the same techniques (one mutation in >300 bp compared to one mutation in >1000 bp) and thus much more DNA-sequencing was needed. This error rate is

explained by the toxic nature of the REPP constructs with a strong biological pressure against a functional enzyme.

corrective actions. Oligo building block purification using the 96 well DEAE CIM disk technology at different temperatures has allowed automatic DNA construction and based on longer DNA fragments. This technology goes far beyond the scope of the project, and constitutes an added value for the entire ADRIACELL research.

Problem 4. REPP activities showed batch variations in intra- and inter-Laboratory testing by RTD partners. The most likely explanation is variation of the REPP polypeptide folding and conditional changes in bacterial membrane translocation, including interaction of the cationic PP portion with outer bacterial wall components, such as peptidoglycan. Also, it became evident that more sensitive and robust assays were required.

corrective actions: Several courses of action were considered:

a) co-incubation of bacteria with a molecules capable of impeding PG formation (e.g. penicillin, lysostaphin for S. aureus).
b) masking of REPP by formulation of cationic residues with suitable bio-compatible polymer was immediately implemented and the modified masked REPP showed strong improvement;
c) testing different types of PP, including ones providing active transport into bacteria
d) introduction of antibacterial assays based on luminescence/fluorescence detection of reporter genes

Problem 5 Self promoted uptake of REPPs based on membrane active PP portions does not allow for sufficiently low active concentrations. Analogy with REPPs designed to work on eukaryotic (tumour cells) suggest that receptor-based active transport systems into bacteria would be more effective.

corrective actions: An in depth biological analysis of the activity of Pro-rich PPs has led to the identification of an active transport system into some Gram-negative bacteria by UTSBC

Problem 6. Production of REPPs containing Pro-rich PPs is quite problematic – these are the only REPP constructs amongst hundreds attempted so far not to have useable expression levels, even with the optimised expression systems developed in period 1 of the project.

corrective actions. design and production of an optimized RE portion with N-terminal Cys residue, and semi-synthetic assembly of REPP with synthetic peptide provided by UTSBC.

Problem 7. The project initially aimed to create a custom software package that would control all aspects of REPP synthetic DNA design, from back-translation into DNA starting from a proposed PP sequence, to control of codon-usages for high expression levels, to selection of construction oligos etc. This was to be subcontracted, and integrated with process control software with embedded tracking by HECUS. At the end of P1 however, excellent commercial software became available for synthetic DNA handling, at a very competitive price.

Corrective action taken. ADRIAC has decided not to proceed with custom software creation, and thus not make use of the subcontracting funds.

– Section 2 –

Workpackage Report

Work Package 1

Objectives: the principal goals of WP1 were:

- Selection of Penetration Peptide (PP) classes suitable for antibiotic REPP
- Principles for oligonucleotide based construction of REPP;
- Cloning of RE and PP fusion proteins (REPPs) at the semiautomatic scale
- Design of REPPs to increase productivity folding/activity and stability
- Design of REPPs with effective PP sequences.
- Second generation REPP expression/purification

Progress towards objectives and deviations from the work programme.

a) The Selection of Penetration Peptide (PP) classes suitable for REPP internalisation into pathogenic micro-organisms. At the beginning of the project, RTD partners provided a shortlist of 35 different PP candidates for REPP constructs. All were constructed as recombinant REPP expression vectors, proteins were expressed and subjected to purification. This required:

   i. codon optimisation and asymetric codon design for the two individual subunits of the PvuII homo-dimer;

   ii. improved PvuII expression vectors with flexible polylinker design at the N terminus, the single chain linker region and the C terminal parts enabling a transition from design to production of REPP fusion proteins in a very short time;

   iii. improvement of the single chain version of PvuII by structure based engineering of the subunit-linker;

   iv. optimizing the production strain capable of dealing with the wide range of lethality profiles for REPP constructs (slight delays of Deliverables D2, D3, D4, D5, D7 can be explained by this biological constraint).

   v. As part of the risk management of the project, the effect of extracellular, constitutive or stress induced proteases, causing rapid REPP degradation, were considered, but REPP proteins showed acceptable steady state kinetics with half-lives times in culture supernatant estimated at > 4 hours for *S. aureus* and *P. aerogenosa*.

b) design of REPPs to increase folding/activity and stability

Once producer strains had been identified and optimised, production levels were further increased by i) selecting growth media; ii) a detailed study of growth and induction kinetics; iii) changing cell lysis protocols to homogenization and tangential flow filtration. By these means, production levels were increased 30 fold.

Folding of the single-chain RE was improved by playing with the spacer region connecting the two homologous subunits (Figure 4). At this point, folding is optimal and has allowed the production of crystals of a suitable quality for 3D structure determination. Better folding was reflected in a more potent nuclease activity. The original patented and published REPP has an activity about 80% that of the double-chain, wild-type RE molecule, whereas the newer generations of optimized folding REPP have an activity of 140%.

Improved folding comes with improved stability in solution for the RE portion. The PP portion remained problematic. Some intrinsic stabilization of this portion could be effected by mutational analysis of the sequence. For example Arg to Lys substitutions in combination with Pro and Leu can render PPs more stable in solution.

First steps towards masking of REPPs with biocompatible polymeric substances has also been effected to improve serum stability. This has increased the half-life of REPP prototypes up to 100 fold (Fig. 5). The success of these steps has prompted ADRIAC to accelerate modification and optimization studies even after the termination date of the ET-PA project. More precise details of the latest generation REPPs cannot be provided at this moment as they are currently subject to patenting by ADRIAC.

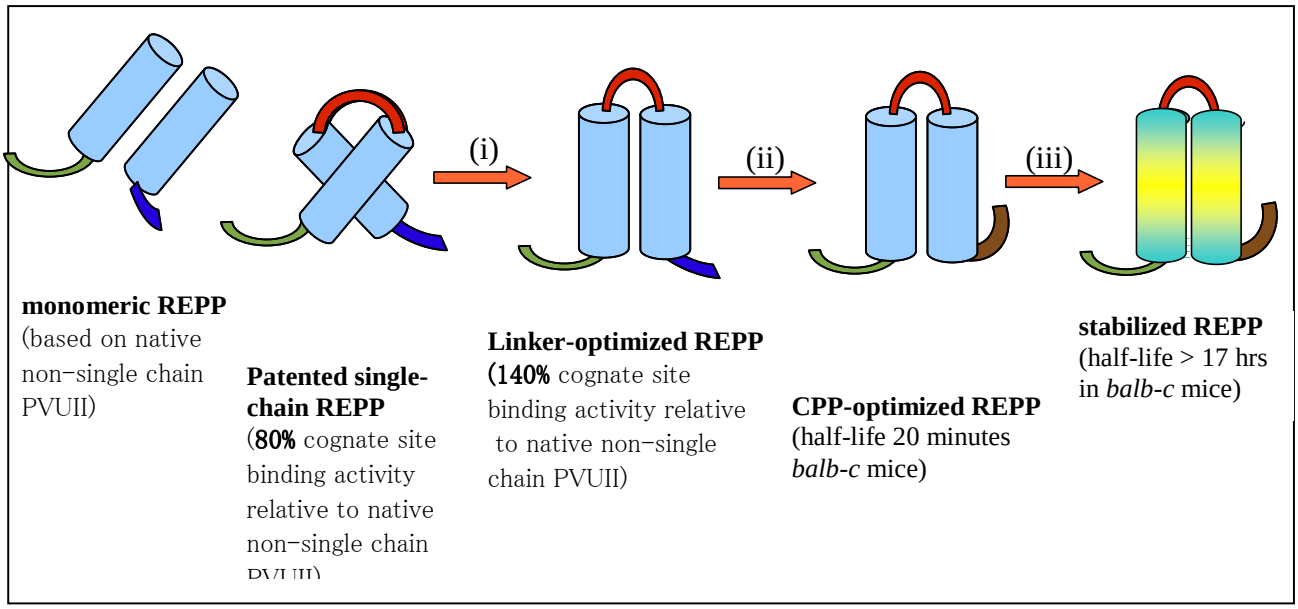

Fig. 4. Structural optimization of REPP architecture: A) Schematic representation of structural variations;
RE domains linker sequence original PP sequence modified PP sequence polymer masking..

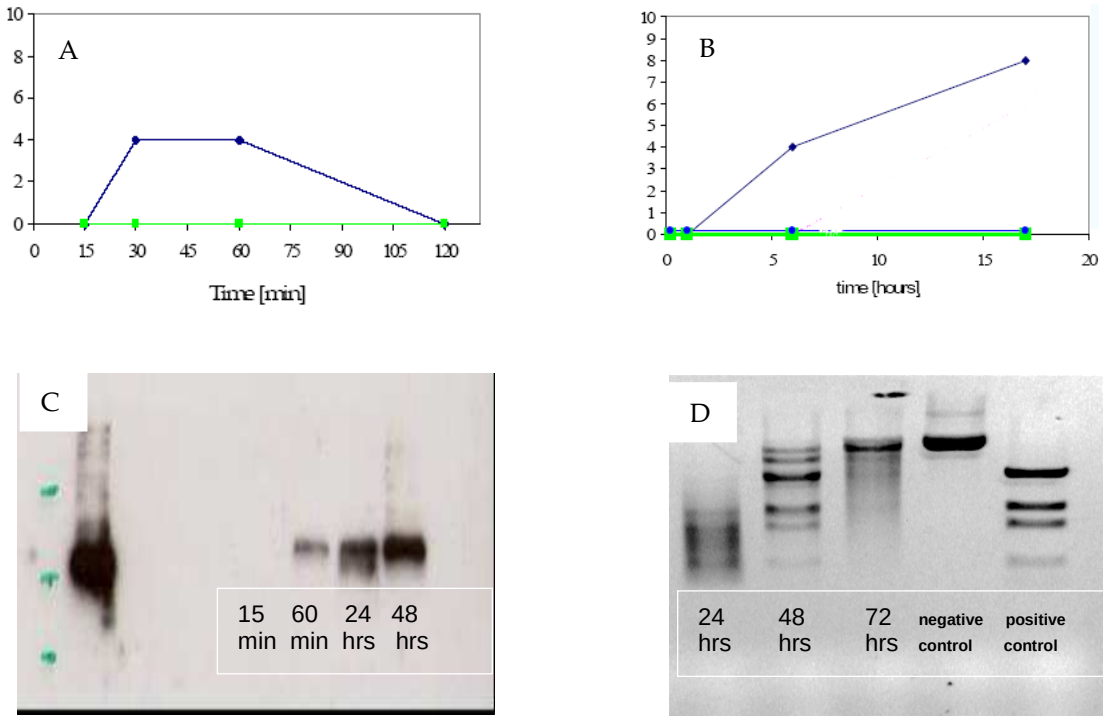

Fig. 5. Optimization of REPP stability *in vivo*: A) example of enzymatic activity of an unmodified REPP in mouse serum (blue) vs mock (green) (after step I in fig 4); B) enzymatic activity of modified REPP in mouse serum (blue) vs mock (green)(after step iii), note that the scale in A is in minutes whereas in B it is in hours; C) Serum levels of optimised REPP after subcutaneous injection in mice; D) Endonuclease activity in serum of optimised REPP, a decrease in RE activity is observed only after 72 hours.

c) design of REPPs with novel PP sequences.

Based on positive results in WP2 (see below) with Pro-rich potential import sequences, ADRIAC has set about designing, constructing and expressing REPPs based on such PP. In order to express these proteins, both *E. coli* expression strains were considered: XL10-Gold (from Stratagene), and BL-21RP (from Novagene). These strains had been optimized in P1 by inserting an expression vector for a methylase that is capable of protecting the host cell DNA against the restriction endonuclease of interest. Furthermore, the BL-21RP strain is optimized also for expression of Pro and Arg codons, so is ideal for Pro-rich REPP. The first two REPPs of this type to be designed contain the bovine Bac7 (sequence 1-35) and the porcine PR-39 (full sequence). Significantly, while the expression systems had both been optimized for high REPP production (at least 15-20 mg/l, see Fig. 6 left), for both these constructs it was not possible to obtain any significant expression, despite using several different growth and induction conditions. For the REPP based on Bac7(1-35), induction was not successful for either strain, while the one based on PR-39, the XL-10 producer cells did not grow while BL-21RP did not express (see Fig. 8 centre and right). It is important to note that for hundreds of different other REPP expression systems studied so far, these are the first two cases

in which it was not possible to obtain expression. We have previously found that for REPPs containing potent lytic AMPs (as for example REPP008 with the SMAP29 PP sequence), expression was lower than optimal (1-2 mg/l) but was in any case evident. A plausible explanation is that the Pro-rich PP REPPs are acutely toxic to the producer strains.

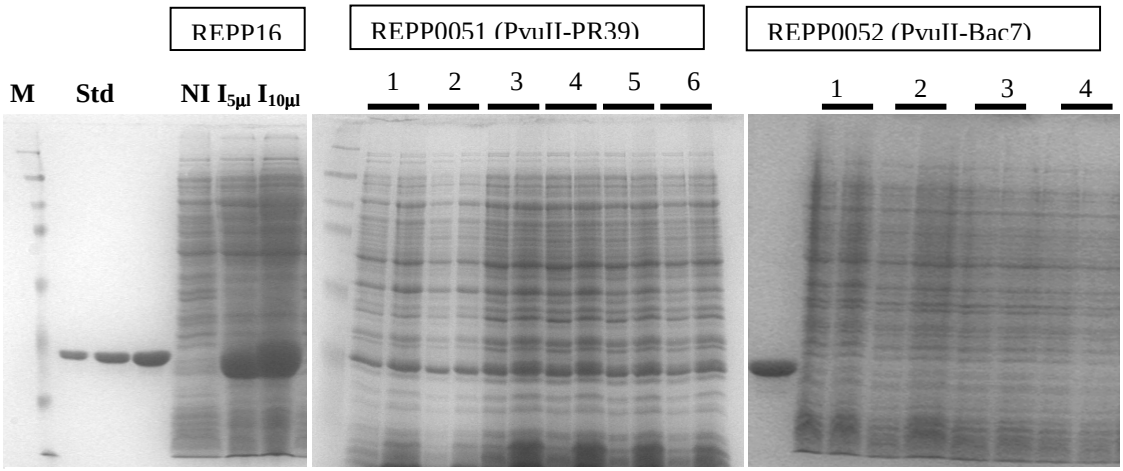

Fig. 6. Expression profiles for different REPP constructs. REPP16 contains the lytic AMP tritrypticine as PP sequences, while REPP0051 and REPP0052 carry the PR-39 and BAc7 Pro-rich PP sequences respectively. Induction of the REPP16 transformed cells results in strong protein expression while induction of the REPP0051 or 0052 transformed systems resulted in little or no expression.

While these results are frustrating, REPPs containing Pro-rich sequences remain of particular interest as they are the prototypes for PP sequences that are actively transported through the bacterial membrane and are not lytic. The problem could derive from REPP released to the extracellular medium and re-internalized via the SbmA transporter. A solution would be to use a producer strain in which the SbmA transport system has been knocked out or rendered inefficient (the necessary plasmids are available with the UTS-BC partner, as used in strain HMM11, see WP2). However, the optimized producer strains are already plasmid saturated and stressed. Alternatively, completely new producer strains intrinsically lacking sbmA could be considered, but this would require a lengthy re-optimization process. The most doable alternative, with which we are proceeding, is a semi-synthetic one. ADRIAC has an optimized RE module bearing a C-terminal spacer stretch ending with a Cys residue. UTS-BC have several Bac7 PP sequences available bearing a C-terminal Cys residue (see Table 1). The recombinant and synthetic sequences are being linked by an optimized oxidization protocol set up at UTS-BC. A further advantage is that these sequences can be fluorescently labeled to allow monitoring of internalization.

A protocol for semi-synthetic RE-Cys-Cys-PP constructs has been set up, using the reduced form of an available REPP protein R3/7 [20638.3Da] carrying a Cys residue at the C-terminal. It was first dissolved in 20 mM sodium phosphate buffer (SPB), 1 mM EDTA, pH 7, containing 150 mM NaCl and 1% of DMSO, resulting in 25 mM final concentration. The reduced B7C was added to this solution in portion wise, resulting in 50 ◎M final concentration. The oxidation was monitored by analytical RP-HPLC and ESI-MS at 2, 5, 22, 28 and 46h. The reaction was not completed by this stage so an alternative folding system was tested, based on addition of the cysteine (2.5mM) / cystine (250 mM) pair. The folding reaction was left over weekend and continuously monitored by analytical RP-HPLC and ESI-MS. At the end of the oxidation the pH was adjusted to pH 2.5-3 and the mixture was centrifuged. The supernatant and the pellet were purified by semi-preparative RP-HPLC (Delta-Pak TM C18, 15 ◎M, 8x100 mm; solvent A: 0.05%TFA in H2O, solvent B: 0.05%TFA in acetonitrile; gradient 25%-65% B in 40 min) and analyzed by ESI-MS (see Fig. 7). The hetero-dimer R3/7-B7C was found in the pellet.

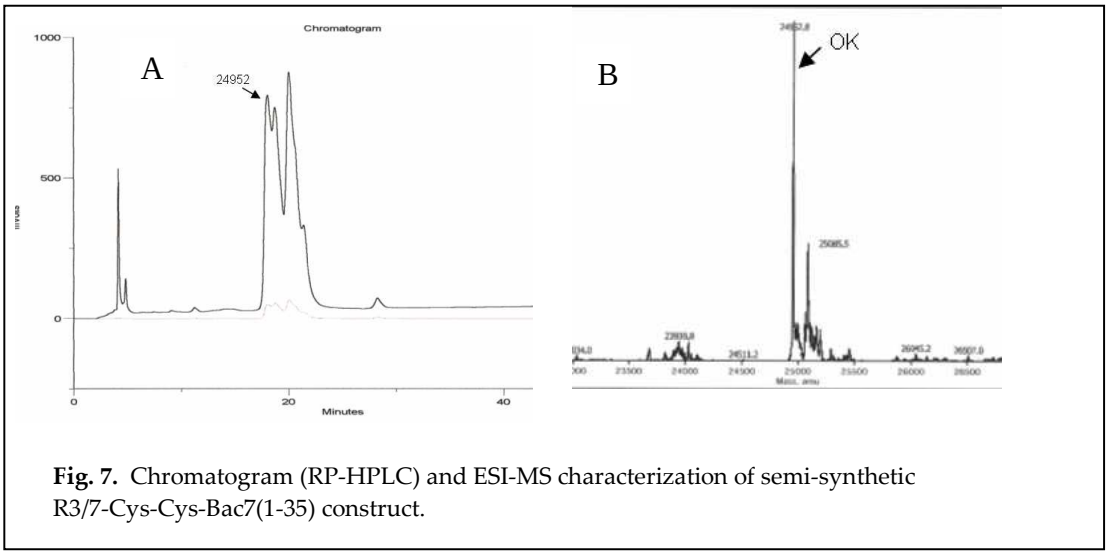

Fig. 7. Chromatogram (RP-HPLC) and ESI-MS characterization of semi-synthetic R3/7-Cys-Cys-Bac7(1-35) construct.

d) second generation REPP expression/purification

Regarding the medium-throughput production process, this can now be carried out almost entirely in the 96 well plates from cloning to the first protein purification step. *E. coli* XL 10 hsdR$^-$ M$^-$ cloning cells are placed in 96 deep-well plates and grown. Lysis is then carried out and plasmids extracted and transferred to custom 96-well format CIM separation plates, where purification is effected. These plates use a 4mm DEAE disc optimised by BIASEP for oligonucleotide/plasmid separation. Purified plasmids are then used to transform the XL-10 producer cells. These are then manually plated and selected, being the only process not involving the 96 well format. Competent colonies are then retransferred to the 96 well format for growth/expression induction. After lysis the supernatant is transferred to another CIM separation 96 plate, in this case with a chemistry suitable for protein separation (affinity method). This provides protein for

subsequent characterization, activity validation and assaying. This technology can easily be transferred to a robotic station for semi-automatic production, should a higher throughput be required. ADRIAC has acquired, and gained experience with, the Hamilton Starlet platform for REPP assays on eukaryotic cells, including reprogramming for process control, which will allow a rapid implementation.

Another aspect that has also been taken into consideration is scale-up for REPP candidates selected for further development. Use of CIM technology in small-scale purification processes, for example using 4 mm discs in 96 well format or in 1 cm discs in column housing format, has proved very effective and warranted scale-up to greater sizes and alternative formats. For preparative scale, 8-80 ml CIM hollow tubes, provided by BIASEP, in appropriate column housings, will be used in conjunction with an Agilent LC-1200 HPLC system, automated with a multi-valve system for HT, and capable of processing litres of supernatant per hour.

Progress towards objectives All tasks required to meet the objectives of the project were carried out as described.

Use of resources. WP1 has made full use of the resources assigned to it. In particular, resources assigned for consumables and equipment were used to acquire perishable lab ware, reagents and consumables for use in REPP expression and purification, solvents and consumables for HPLC, microtiter plates, electrophoresis reagents, restriction enzymes.

WP1, for this reporting period required 113 out of the total of 113 person months for WP1 during ET-PA. In particular, the ADRIAC partner provided 78 person months from both company and EU-funded personnel, mainly to assist in preparation and optimisation of REPP constructs. Biaseparations provided 9 person months from both company and EU-funded personnel, mainly to application of CIM technology to REPP purification. Hecus provided 10 person months from both company and EU-funded personnel, mainly to assist with automatic REPP construction and to study applications for the thermo device. UTS-BC provided 12 EU funded person months.

WP1 - Person-Month Status Table

CONTRACT Nº:	COOP-CT-2005-018191	Partner - Person-month WP1							AC – non EU paid		
ACRONYM:	ET-PA										
PERIOD:	1/08/2006 -29/02/2008	TOTALS	Coord.	Partic. 1 ADRIAC	Partic. 2 BIASEP	Partic. 3 HECUS	Partic. 4 UTSBC	Partic. 5 UBIMMI	AC TOTALS	AC partic. 4 UTSBC	AC partic.5 UBIMMI
Design/ Production	Final WP total:	113	2	78	9	10	12	2	5	3	2

WP1 List of deliverables due in reporting period:

D2 First 10 REPP recombinant protein expression constructs

D3 REPP CIM disk monolythic purification principle

D4 First 10REPP recombinant proteins, manual scale

D5 REPP construction computer software program (transferred to WP3)

D8 REPP CIM disk monolythic purification principle semi-automatic scale

D10 REPP recombinant protein expression construct library (semiautomatic-scale)

D12 Computer software, installation and documentation (not implemented)

D13 REPP recombinant protein library (semiautom-scale)

Work Package 2

Objectives: the principal objectives of this work package for the project were:

- Screening different REPP constructs for antimicrobial activity
- Relating antibiotic activity to the cell internalizing capacities of the PP domain
- Characterizing the microbial strain phage background
- Evaluating penetration peptides (PP) suitable for internalization of cargo into bacteria, and identification of possible active import mechanisms into bacteria
- Evaluation of bacterial targets
- Developing of more sensitive and robust assays suitable for higher throughput screening
- Testing of second generation REPPs

a) Screening of REPP activity. This required the implementation of suitable assays. An initial rapid assessment of antimicrobial activity was carried out using an automated Tecan Sunrise 96 well plate reader to provide growth inhibition data in liquid medium (see P1 report). Different conditions were tested (e.g. medium composition, exposure times etc.) so as to define those that best ensured reproducible results. This system has the advantage of being semi-automated, allowing the testing of small amounts of several different REPP constructs, against different bacterial species, under identical conditions. REPP contructs were also subjected to a time-killing assays on solid medium, which allows determination of the decrease in bacterial population with time, in terms of counted colony-forming-units. The more promising REPP candidates were independently tested using a NCCLS standardised MIC assay on drug-resistant clinical bacterial isolates. Staphylococcal strains were also analyzed as to phage background to obtain information on the presence of phage-mediated restriction-/modification enzymes. No indication was obtained that would point to an interference of such enzymes with PvuII.

b) Relating antibiotic activity to the cell internalizing capacities of the PP domain. This initially required setting up suitable assays. To this end, it was decided to initially study cell penetration by the PP (penetration peptide) moiety alone. Three different classes of peptides were selected for synthesis: the membrane-self-internalizing non-lytic peptide Bac-7, the cell-lytic peptide LL-37, and the membrane translocation domain of the Pep5 bacteriocin immunity peptide. All peptides were provided with a C-terminal cysteine to allow for labelling with the fluorescent probe BODIPY. Chimeric peptides were also synthesised by fusing penetration peptides reported to internalise into *E.coli* or *S.aureus*, respectively, with the antimicrobial domain of Bac7 stripped of the internalization promoting sequence. Membrane localising polymixin, labelled with BODIPY, is used as control. Flow cytometric experiments using the Bac-7/BODIPY system in the presence of fluorescence quencher clearly show peptide internalisation into bacterial cells. Extensive mode of action and SAR data have been obtained for potential penetration peptides LL-37 and

Bac-7 peptides as representative of non-lytic and membrane-active PPs. Genetic studies to characterise the LL-37 gene in non-human primates, performed by UTS-BC, has allowed the definition of structural analogs, thus permitting a detailed SAR study (Zelezetsky et al., J. Biol. Chem., 2006). Extensive biophysical studies are being carried out to determine the mode of interaction of these peptides with model and microbial membranes using both biophysical (CD, FTIR, dye release assays) and biochemical (bacterial membrane permeabilization and depolarization assays, flow cytometry) methods (Morgera et al., Biochem. J., 2008). An extensive SAR study has also been carried out using Bac7 and truncated analogs, as well as its all-D enantiomer. These have conclusively demonstrated that non-lytic internalisation of the peptide depends on the stereochemistry and require energy and a strong membrane potential, pointing to a mediated translocation. Data generated from these studies that is considered non-sensitive, is being disseminated after clearance from SME partners.

d) identification of possible active import mechanisms into bacteria

During early stages of the ET-PA project, it became apparent that the novel REPP class of protein antibiotics showed distinct pharmacokinetics from conventional small molecule antimicrobial agents or from canonical host defence peptides (AMPs). These differences presented the risk of high drop out rates during first screening rounds within the REPP screening program and it became evident that the internalisation mechanisms had to be better characterized. Passive membrane translocation by some penetration peptides (PP), based on membrane-active antimicrobial peptides, was effective for some pathogens but not others. In a parallel program for developing REPP-like molecules for internalisation into tumour cells, activities orders of magnitude higher have been achieved, likely due to receptor-based internalisation. The UTS-BC partner has recently identified a component of the putative transport system for Pro-rich AMPs into some Gram-negative bacteria. This type of antimicrobial peptides (AMPs) is in fact not membranolytic, but rather subject to active transport into susceptible bacteria where the peptides then interact with intracellular pathogens [1-3]. The Pro-rich peptides produced by some mammals can have considerable sizes (up to 100 residues), but only relatively short N-terminal sequences are necessary for transport, so that they could act as effective PP for protein cargo. Furthermore, this type of PP is particularly effective towards bacteria such as *E. coli* against which REPP constructs tested so far resulted ineffective.

The transport system for Pro-rich AMPs was recognized by a genetic method based on identification of bacterial genes that, if modified, result in decreased AMP susceptibility. Amongst these was the *sbmA* gene coding for a transmembrane protein involved in the uptake of various peptides [2]. This protein was found to be the transmembrane domain of a putative ABC transport system, and we have shown it to be involved in translocation of several different types of Pro-rich peptides. In particular, by using such AMPs labelled with the fluorescent molecule BODIPY, we were able to show that these were internalised without membrane lysis, as they were no longer accessible to an extracellular quencher, while the membrane remained impermeable to extracellular probes (Fig.8).

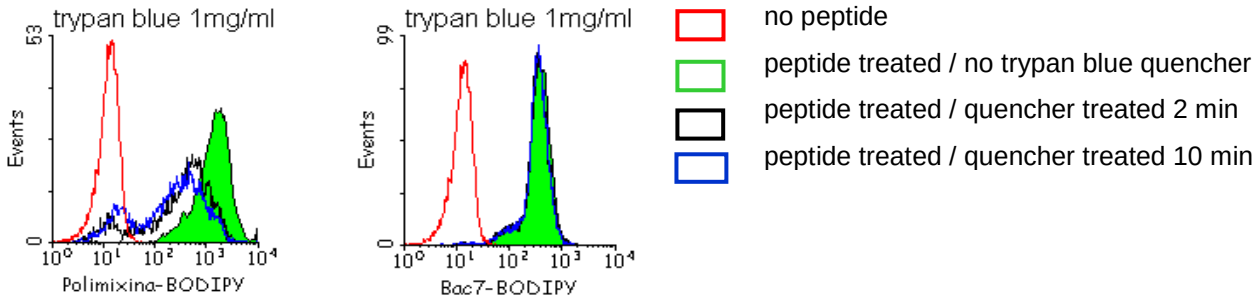

Fig 8. Flow cytometric analysis of bacterial cells (*E. coli* HB101) untreated (low fluorescence, red band) or treated with either BODIPY labelled polymixin (left, a membrane active AMP) or BODIPY labelled Bac7 (right, a Pro-rich AMP). In both cases, bacterial cells became fluorescent after treatment (green bands). Addition of the quencher trypan blue significantly quenched polymixin-treated cells (indicating that this was present on the surface of the membrane and thus accessible), while it hardly affected fluorescence of Bac7-treeted cells (indicating that the peptide had been internalised and become inaccessible). Washing cells with high salt concentrations did not affect results, also indicating that Bac7 was not simply membrane associated. Treatment with propidium iodide showed that cells had intact membranes, and thus that internalization was effected without lysing the membrane.

A structure-activity relationships study was carried out and is continuing, to help to identify the putative internalisation sequence in a bovine Pro-rich peptide, Bac7 (Table 1). This likely covers the first 16 residues from the N-terminal and represents a potential PP sequence for active transport of cargo into Gram-negative pathogens that express *sbmA*-like genes. Variants of the Bac7 have been designed and fluorescently labelled to further characterize this and other import mechanisms (see P2 report). In particular, variants were made which i) either lack part of the putative N-terminal internalization sequence, ii) where this is replaced by other published CPP sequences reported to effect translocation into different types of microbial cells, likely by other means, or iii) where key residues have been modified to determine their importance.

Testing has been principally against gram negative pathogens expressing the sbmA transport system (*E. coli* ATCC25922 , HB101 and HMM11 and *S. thypimurium* ATCC14028 although some peptides were also tested against as *S. aureus* (see P2 report). The presence of the 4 terminal residues RRIR in particular is necessary for both activity and internalization. It is interesting to note a more potent activity correlates with expression of active sbmA, so that *E. coli* ATCC and HB101 strains and *S. thypimurium*, which express *wt* sbmA are all more susceptible than HMM11 strain, which has an sbmA variant less effective in transporting Pro-rich peptides. Altering the two N-terminal arginine residues was effected by either asymmetric dimethylation of the ω amine group of the guanidine, or inserting a nitro group on the imino nitrogen. In the

first case, the pKa and H-bonding capacity is reduced, while size and hydrophobicity increased. In the second case, the charge is reduced while H-bonding capacity and size are maintained. Results indicate that size and H-bonding may be the key factors, consistent with a receptor-linked uptake. Preliminary experiments furthermore indicate that the N-terminal stretch cannot be replaced with other types of import sequences[FF-, NP- and VLBac7(5-35)-Cys] although work is in progress to determine whether this is due to less efficient import or to a reduced capacity to interact with the internal target.

The *sbmA* gene has now been cloned into the pQE9 expression vector and used to transform the *E. coli* XL1 strain, which now over expresses the protein. Cell aliquots were collected at 0, 2 and 18 h after IPTG induction and analyzed both for expression of SbmA by western blot analysis (Fig 6) and increased susceptibility to Bac7(1-35) by MIC value determination (Fig 7). Overexpression of *SbmA* effectively leads to an increased susceptibility to Bac7(1-35), indicating that it is more efficiently internalised. This strain is ideal for testing REPP constructs containing Pro-rich PP sequences.

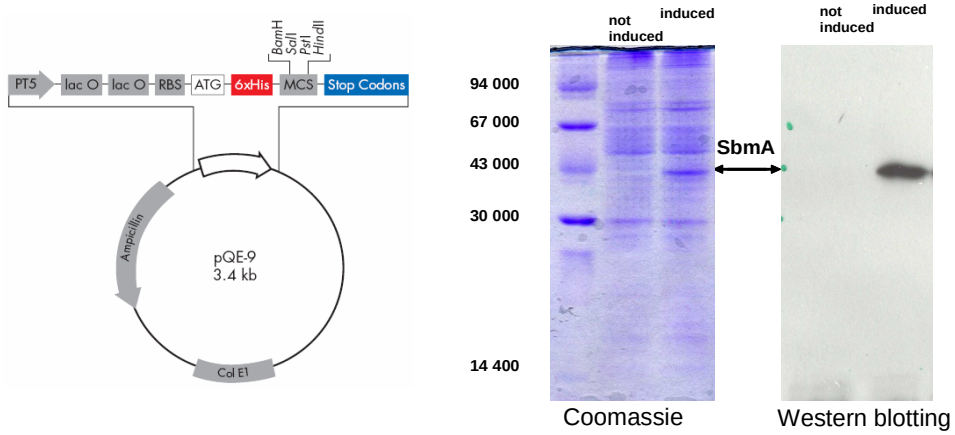

Fig. 6 sbmA cloning into E. coli XL1 strain. The Plasmid vector used for transformation of E. coli XL1 strain with sbmA gene was the pQE9 vector (gene inserted into MCS cassette). IPTG was then used to induce expression, as indicated both by normal staining or western blot with anti His tag antibodies.

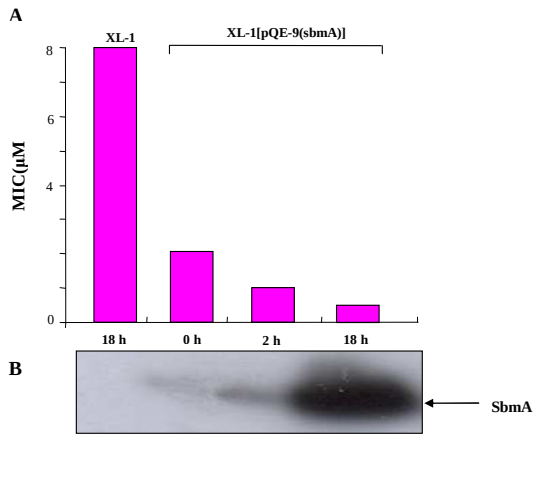

Fig. 7 sbmA overexpression in *E. coli XL1* strain results in increased susceptibility to Pro-rich PP. MIC values decrease from 8 to 0.5 after 18h induction with respect to the untransformed strain

The UTS-BC group also continued to study the mode-of-action of membranolytic peptides, and in particular, that of the human AMP LL-37, which showed promising antimicrobial activity when fused into REPP constructs. A detailed biophysical and biochemical assessment of LL-37 and several primate orthologues has been carried out [4,5]

d) evaluation of bacterial targets for REPPs.

For the initial screening of REPPs, a set of 5 strains were used: four representative multiresistant strains (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecium*) and one standard laboratory strain (*Micrococcus luteus*) which was sensitive to a large range of antimicrobial peptides. The most likely explanation is variation of the REPP polypeptide folding and conditional changes in bacterial membrane translocation, including interaction of the cationic PP portion with outer bacterial wall components, such as peptidoglycan. MIC determinations revealed activity for some of the REPPs against *M. luteus,* whereas for the SMAP-based REPP against the *S. aureus* 8325 strain. Killing kinetics at 8x MIC a continuous decrease of about 3 log in the number of colony forming units (cfu) over 21 h was observed. Results were not always consistent between the two RTD labs, prompting introduction of more sensitive and robust assaying methods (see below).

As PPs present in active REPPs such as that derived from the highly membranolytic AMP SMAP-29 could provide par of the killing action, it was necessary to determine whether activity was due to the REPP itself or to the lytic activity of the PP. Measurement of the membrane potential across the cytoplasmic membrane of bacterial cells (see P2 report) showed that this was not effected indicating that the cidal activity of the REPP does not derive from the lytic PP portion, but rather may derive from translocated RE.

To investigate whether REPP antibacterial activity against the tested clinical strains is decreased by restricted transfer across the cell wall, activity assay was established also using *Mycoplasma*, which lacks cell walls. *Mycoplasma* cells are similar to protoplasts, but they are more resistant against osmotic lysis due to the presence of sterines in the cytoplasmic membrane (see P2 report). An indirect assay was established, in which growth was determined on the basis of colour changing units (ccu). Significant activity for REPPs were however not found against Mycoplasma. REPPs with hybrid bacterial/eukaryotic cell targeting PPs are now being considered.

e) Development of more sensitive and robust assays suitable for higher throughput screening - Standard microdilution assays and time-killing assays were found to be too labour intensive and slow, while automated plate reading based on scattering/absorption was not sufficiently sensitive and difficult to adapt in its current form for multiplexing. With the coordinator's approval, funds set aside for equipment in P2 were used to co-fund fluorescence/ luminescence/fluorescence polarisation plate-reading equipment with

which to develop more sensitive assays to resolve the above constraints, based on appropriate luminescent/fluorescent bacterial reporter strains (see P2 report)

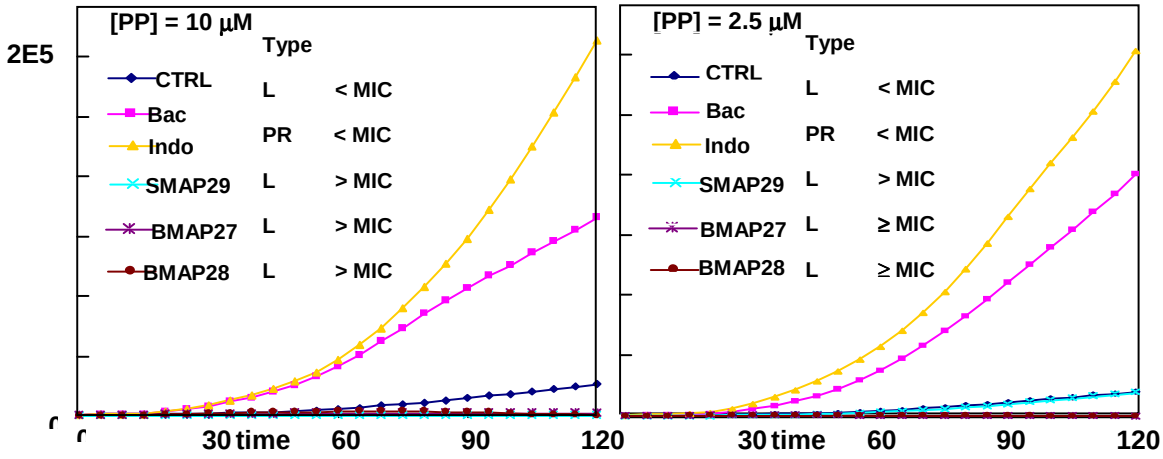

Fig. 6 Bacterial growth inhibition assays based on luminescent P. aeruginosa. Unchallenged bacteria show an increase in luminescence which is evident about 30 min after sowing about 10^5 CFU bacteria in 96 well plates (Chamaleon reader, λ = xxx nm). Challenge with selected PP sequences resulted in different effects depending whether the peptides were present at a concentration above the minimum inhibitory concentration for P. aeruginosa (indicated as > MIC), in which case no luminescence increase was observed, or at sublethal concentrations (< MIC) in which case the bacterium seemed to ramp up its metabolism resulting in string luminescence increase. Y axis presents Fluorescence integrated.

The luminescent reporter strain has initially been tested using numerous sequences chosen from among the PP considered for REPP construction. The assays provided more information than expected, which represents an advantage but also requires more extensive tuning and understanding of the system before it can be applied to REPP testing. In fact, while at high PP concentrations (several times the MIC), the expected decrease in luminescence was observed, at lower concentrations (at or below the MIC), an initial strong increase in fluorescence was observed. This likely derives from the bacterial cell reacting to antibiotic offence by ramping up parts of its metabolism. It is necessary to better understand these aspects, and also to determine the most stable assay conditions for maximum reproducibility. Once the assays are completely defined, the advantage is that by performing them at sublethal REPP concentrations, information can be gathered on bacterial reaction to offence, and help develop methods to overcome these, thus helping prevent resistance development.

The next step will be to transfer the Tn5-luxCDABE cassette to other bacteria, in particular to the *E. coli* strain over-expressing the sbmA Pro-rich AMP transport machinery, so that the assay can be more generally used, as well as perfecting fluorescence-based assays for REPP activity against Gram+ bacteria.

References:

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4) F. Morgera, L. Vaccari, L. Creatti, N. Antcheva, A. Tossi (2008) "Structure and activities of AMPs at the bacterial membrane" in Membrane-active peptides: methods and results on structure and function. M. Castanho Ed. IUL Publishers (International University Line), La Jolla, California, USA (in press).
5) F. Morgera L. Vaccari, N. Antcheva, S. Pacor, A.Tossi (2008) "Differential structuring and membrane interaction modes of primate Cathelicidin orthologues" (manscrpt in preparation)

Progress towards objectives All tasks required to meet the objectives of the reporting period were carried out as described.

Use of resources During this period, WP2 has made full use of the resources assigned to it for the second period. In particular, resources assigned for consumables and equipment were used to acquire perishable labware and media for microbiological assays, equipment to aid mass-spectrometric characterization of REPP constructs and synthetic penetration peptides, reagents and consumables for use in automated peptide synthesis and purification, and solvents and consumables for HPLC, microtiter plates, pulse-field-gel-electrophoresis, restriction enzymes.

The WP2 required all 133 person months planned for this reporting period. In particular, the ADRIAC partner provided 6 person months from company funded personnel, mainly to assist in preparation of REPP constructs for assaying, REPP assay setup or assist with HPLC and mass-spectral analyses of REPP constructs. HECUS invested 2 person month as projected and BIASEP 1 person month to assist assay evaluation. UBIMMI provided 54 person months, technical staff and students (30 person months) and EU-funded personnel (24 person months). UTS-BC provided 68 person months, of which 55 EU-funded person months.

WP2 - Person-Month Status Table

CONTRACT Nº:	COOP-CT-2005-018191		Partner - Person-month WP2							AC – non EU paid		
ACRONYM:	ET-PA											
PERIOD:	1/08/2006 -29/02/2008		TOTALS	Coord.	Partic. 1 ADRIAC	Partic. 2 BIASEP	Partic. 3 HECUS	Partic. 4 UTSBC	Partic. 5 UBIMMI	AC TOTALS	AC partic. 4 UTSBC	AC partic.5 UBIMMI
	Evaluation	Final WP total:	133	2	6	1	2	68	54	43	13	30

WP2 List of deliverables:

D6 Assessment table of REPP activities (First 10 REPP constructs) (month 9)

D9 Short-list of active REPP constructs (month 12)

D14 Initial assessment tables for REPP libraries (month 28)

D15 Efficacy/cytotoxicity data table (month 30)

Work Package 3

Objectives: the principal objectives of the work package 3 for project were:

- Flow diagram for REPP vector construction, protein expression and purification for final implementation into the process controlled technological platform
- Comprehensive proof of concept for the CIM multi well implementation
- Robotic platform solution for production and/or assaying REPPs
- Interfaced thermo-device for specific CIM/REPP as well as for broad range applications

Progress towards objectives All tasks required to meet the objectives of the reporting period were carried out as projected. Additionally, added value was created by broad range applications of the termo device beyond REPP technology.

a) Flow diagram: A flow diagram that describes the elaborated techniques for REPP vector construction, protein expression and purification from WP1 was designed in a common effort and serves for implementation into the process controlled platform. This work was based on the principles elaborated from WP1 and provides a flexible and semi-automatic process for the production of protein libraries in general and REPP libraries in particular. Apart from the specific goals of the ET-PA project, to provide a versatile REPP construction and screening platform, this can also be used for bio-pharmaceutical screens in general, and has a high potential for creation of additional value for the SME's involved.

b) Comprehensive proof of concept for the CIM multi well implementation - For the REPP production process in either manual or semi-automatic processes, CIM disc technology was adapted for 96 multi-well formats (96CIM). This techniques is now routinely used in the ADRIAC processes to purify olgonucleotides and proteins, not only for REPP synthesis within the ET-PA project, but in general for all molecular biology applications. A proof of principle for suitability of production of the more complex 384 well format has also been obtained by BIASEP (IP BIASEP not included into the project). Along these lines thus during the ET-PA project, BIASEP implemented most of its marketed CIM chemistries into the 96 well format. This included various Ion exchange (DEAE, QA, SO3) and chelating principles (Ni^{++}, Cu^{++}, Co^{++} IDA). Prototypes of these devices were tested by ADRIAC for REPP protein separation. More than 20 96CIMs were produced and validated by BIASEP production analytic and REPP applications are now in the BIASEP catalogue.

c) Robotic platform solution for production and/or assaying REPPs - In designing the ET-PA project a strong emphasis was placed on the custom design of in-house software for semiautomatic REPP design that should not only control and operate but also track all the individual passages. This in turn was expected

to give the ADRIAC and HECUS partners an additional competitive advantage for their value added chain, establishing a solid platform for REPP production for ADRIAC and the possibility to enter into the biotech market for HECUS. During ET-PA this was revised due to the actual needs that resulted from the progress in WP1 and WP2, but also by the strong growth of both companies in other areas, during this period. During the course of the ET-PA project, however, a new generation of commercial roboting workstations became available which offer these features off-the shelve. ADRIAC has acquired a roboting work station, principally for its tumor therapeutic program, which was however also used within the ET-PA project. Although none of the costs for this acquisition were allocated to the ET-PA project, special upgrades of the software were acquired and programmed also for the needs of the REPP production. HECUS was involved to adapt these devices for REPP research instead rather than custom programming and put more effort in the added value application for the custom thermo device that was brought to a marketable product.

d) Interfaced thermo-device for specific CIM/REPP as well as for broad range applications - A temperature controller was planned to be developed for temperature control of Multiplex CIM applications. However, multiplex applications proved to be too complex as individual proteins were not independently accessible for temperature control studies in the 96 well format. The coordinating company for the thermo-device, HECUS, however decided proceed with design and development generic temperature device that now ads value to the key business of HECUS GmbH. (http://www.hecus.at/index.php?open=ASA_PGS; http://www.hecus.at/index.php?open=Pharmaceuticals; http://www.hecus.at/index.php?open=Proteins). For this reason, D7 was renamed to "D7 Thermo custom device for general use". Concerning the ET-PA project, this device will be useful for studies on single CIM 0,35 cm discs, were temperature can be used for the separation of synthetic DNA as well as for carefully optimized protein refolding projects (preliminary data not disclosed). The temperature controller unit is now in the product catalogue of HECUS and is described in detail in the P2 report.

Use of resources During this period, WP3 has made use of the resources assigned for personal costs, and consumables and travel costs and projected costs for equipment. As WP3 was strongly interactive with WP1 during the entire ET-PA project visits at HECUS, BIASEP and ADRIAC by the participants were needed.

The WP3 required 72 person months as anticipated for the project. In particular, the HECUS partner provided 35 person months (EU as well as company funded), mainly to set up and document the thermo control PID unit and for REPP program development with ADRIAC. ADRIAC provided 6 person month (company funded) and BIASEP used 30 person month for completing CIM multiplexing (EU as well as company funded)

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WP3 - Person-Month Status Table												
CONTRACT Nº: COOP-CT-2005-018191		Partner - Person-month WP3							AC – non EU paid			
ACRONYM: ET-PA												
PERIOD: 1/08/2005 -031/07/2006		TOTALS	Coord.	Partic. 1 ADRIAC	Partic. 2 BIASEP	Partic. 3 HECUS	Partic. 4 UTSBC	Partic. 5 UBIMMI	AC TOTALS	AC partic. 4 UTSBC	AC partic.5 UBIMMI	
Enabling Technologies	Final WP total:	72	1	6	30	35	0	0	0	0	0	

WP3 List of deliverables due in reporting period:

D5 Construction computer software program (REP-CSP)

D7 Thermo custom device for general use

D11 20 x CIM® disks specific for REPP purification in the 96 well format

D12 Computer software, installation and documentation

Work Package 4

Objectives: the principal objectives of the work package 3 for project were:

- Project web page
- Project Coordination
- Fund raising from public and private bodies, company expansions and added values.
- Exploitation assessment report and dissemination plan.

Progress towards objectives: WP4 proceeded on schedule throughout the project

a) **Project web site** - The ET-PA project web-site was established (www.et.pa.org), for dissemination of the project and to stimulate communication within partners and affiliates;

b) **Coordination** - The coordinator of the project, from ADRIAC, with support from the other partners, was heavily involved in ensuring an adequate level of cross-talk between partners, in collecting and collating all information necessary for reporting and assembling of deliverable descriptions, and finally for the exploitation assessment report and dissemination plan. Project coordination did not present problems as the partners networked effectively.

c) **Fund raising** - from public and private bodies, company expansions and added values**.** First contacts were initiated already in the first period of the project towards fund raising from public or private partners for further development of the REPP technology for intracellular applications, and for finalizing respective topical ET-PA REPP formulations resulting in an IND application. These efforts were aimed to bringing REPP into clinic testing, and the "96 CIM disk" technology and the Hecus thermo device directly to the market were followed independently by the three SMEs partners, mainly based on the companies internal business models. Many disclosable issues were consulted with the other participants during specific meetings.

A shortlist of contacts from public or private partners was assembled during the second period of the project by the individual SME partners towards fund raising for further development of their proprietary technologies. Participation in the ET-PA project was of significant importance towards this fund-raising. For ADRIAC, this was carried out with strong support from UTS-BC. In particular, ADRIAC and UTS-BC have raised public funds with the Friuli-Venezia-Giulia region in which it is located, in projects related to the development of the REPP concept for antitumor applications. ADRIAC also finalized a very successful A round financing with both an Italian and a German venture capital fund. This was possible due to the increase in the pre-money valuation of ADRIAC during this period deriving in part from the impact of ET-PA project, which was perceived as an advantageous added-value for the company. BIASEP underwent a very successful restructuring and consolidation period, and has closed a venture equity financing round for relocation to larger premises. Cooperation with Agilent Technologies (http://www.biaseparations.com/news.asp?FolderId=241&ContentId=1171) for the analytical bio-monolithic technology market confirms BIASEP as the world leader in the Monolitic Device market. HECUS expanded by acquisition of complementary technical products on taking over the PSD detector

business branch of M. BRAUN, Munich (http://www.mbraun.de/), and was refinanced by private equity from a silent partnership. HECUS has not only contributed to implement the semiautomatic REPP production platform for ADRIAC but has created application friendly softwares for their products and added the developed thermo device for broad range applications to its catalogue. During the ET-PA period HECUS has thus made the big step from an instrument producer for highly specialized clients to a more user friendly application specialist.

Besides substantial equity financing rounds for all there SMEs during this period a further index of the success of the three SMEs has been their expansion after the start of the project in terms of personnel, with positive effects in employment creation as summarized in Fig7. below. Naturally, this was not exclusively due to ET-PA. However the direct outcomes as described in this report and also the network created that will last beyond the duration of ET-PA provides for a critical mass essential for ADRIAC's future, and down stream processing applications might well involve BIASEP and HECUS as CRO for GMP production and formulation analytics, respectively.

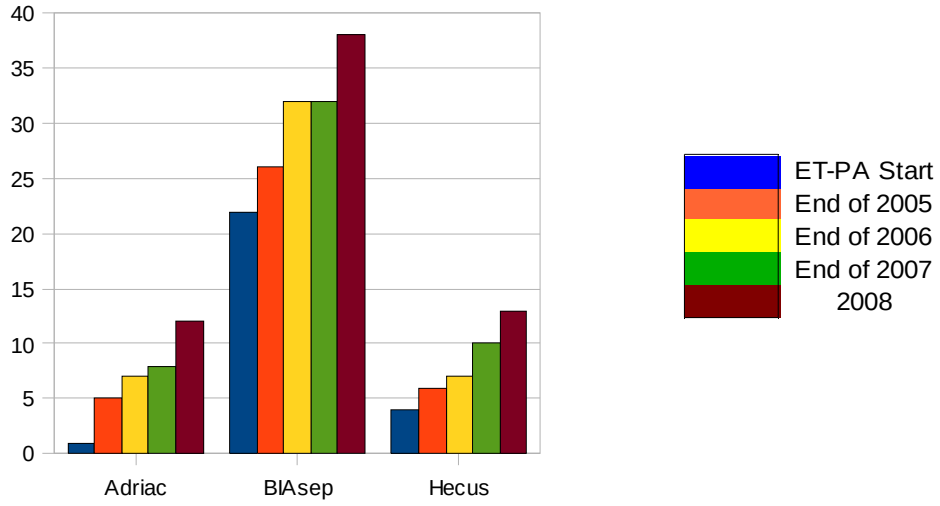

Fig 7. Growth of employment in the three participating SME's during the period of the ET-PA collaborative project;

d) Exploitation assessment report and dissemination plan - In terms of exploitation the ET-PA project was very successful: one SME, ADRIAC, is continuing into preclinical trials with knowledge deriving from the project (bioavailability of REPP proteins); the second SME, BIASEP, is entering the analytical bio-monolithic technology market with its Monolitic Devices, including 96 well CIM plates, and is opening new production units; the third SME is expanding and entering the biomedical market, converting from an instrument producer for highly specialized clients to a more user friendly application specialist. The "Exploitation assessment report and dissemination plan" are presented as deliverable D16.

Progress towards objectives All tasks required to meet the objectives of the reporting period were carried out as described.

Use of resources. During this period, WP4 has made use of the resources assigned for travel costs. Personal costs for SME were funded directly by the companies. The University partner UTS-BC was very actively participating in WP4 and the personal costs were EU funded. This was agreed by the consortium, as due to overlapping interests with other company objectives WP4 required more person month resources as planed but extra resources however were difficult to separate by means of ET-PA and non ET-PA priorities. Thus WP4 required 37 person months, subdivided as 20 for ADRIAC, 7 for HECUS, 8 for BIASEP and 2 for UTS-BC. Note that the increase in person month is mainly due to the increase in involvement of all the SME partners in contrast to the projected person month resources. This also reflects the importance of management of research in market based environments - clearly underestimated at the planning level, however most of the personal costs for this package were born by SME partner internal resources and result as such cost neutral. All company person months were company funded.

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WP4 - Person-Month Status Table													
CONTRACT Nº: COOP-CT-2005-018191			Partner - Person-month WP4							AC – non EU paid		
ACRONYM: ET-PA													
PERIOD: 1/08/2006 -29/02/2008			TOTALS	Coord.	Partic. 1 ADRIAC	Partic. 2 BIASEP	Partic. 3 HECUS	Partic. 4 UTSBC	Partic. 5 UBIMMI	AC TOTALS	AC partic. 4 UTSBC	AC partic.5 UBIMMI
	Management	Final WP total:	37	0	20	8	7	2	0	2	2	0

WP4 List of deliverables due in reporting period:

D1 ET-PA project website

D16 Exploitation Assessment Report and dissemination Plan

D17 Exploitation Report Digest Hard and Web copies

Deviations from the project work programme and corrective actions taken.

Regarding WP1, all activities were very successful and there were no substantial deviations from the workplan. The initial monomeric RE concept, proprietary to ADRIAC, was necessary to allow efficient cellular import, but suffered from low production yield, lower activity than the *wt* RE, due to inefficient folding, and insufficient serum stability for useful *in vivo* activity. At the end of the ET-PA project producer strains had been optimised to increase yields almost 100 fold, folding problems had been resolved allowing to pass from an 80% the *wt* RE activity to over 140%, and serum stability taken from minutes to days. The only production problem encountered was with a single REPP class, that based on Pro-rich PP sequences. This is frustrating as it is a potentially usefull class (see below). Corrective action taken, in collaboration with UTS-BC is development of a semi-synthetic protocol for covalent assembly of a specifically designed recombinant RE-Cys with a chemically synthesised Pro-rich PP-Cys sequence.

Regarding WP2, RTD partners carried out antimicrobial activity tests to provide proof-of-concept for antibiotic REPPs. Activity was effectively found for some REPP constructs during P1, which was encouraging, but it was not as high as expected and too dependent on medium conditions and the bacterial batches used. Extensive discussions at the end of P1 led to a consensus that this was likely due to erratic penetration by REPPs. The Penetration Peptides used for REPP construction had been based mostly on membranolytic antimicrobial peptide sequences, by far the most common known, and clearly the self-promoted uptake these were likely to engender was not a sufficiently robust penetration method. In contrast, REPP type molecules with PP internalization sequences for host cells, developed by ADRIAC in a parallel antituymour programme, acting by a receptor-based mechanism, led to low-nanomolar active concentrations. The main corrective action taken for antibiotic REPPs was an in-depth analysis of a possible active transport system into Gram-negative bacteria, which has been shown to internalise specific Pro-rich PPs. Concomitantly, some effort was expended in developing novel, more sensitive antimicrobial assays, and identifying potential new bacterial targets, such as intracellular pathogens devoid of cell walls. These efforts, as well as those in WP1 required an extension of the project from 24 to 31 months, which partners readily agreed to without requesting any funding increase.

Concerning WP3, the project initially aimed to create a custom software package that would control all aspects of REPP synthetic DNA design (from back-translation into DNA starting from a proposed PP sequence, to control of codon-usages for high expression levels, to selection of construction oligos etc), that was to be subcontracted. This software was to be integrated with process control software with embedded tracking by HECUS. At the end of P1 however, excellent commercial software became available, at a competitive price, for synthetic DNA handling. ADRIAC has thus decided not to proceed with custom

software creation and thus not to make use of the subcontracting funds. HECUS aided Adriac in doing this and in addition put more effort into the now marketable thermo device.

Concerning WP4, all management activities proceeded without significant deviations from the work-programme. Dissemination was adequate and fund-raising activities

Project Deliverables and Milestones Lists

Table 1: Deliverables based on a seven month project extension

Del. no.	Deliverable name	WP NO.	Person-months	delivery date (month)	Lead partici-pant
D1	Project web-site	4	2	4	ADRIAC
D2	REPP recombinant protein expression constructs (10)	1	20	6	ADRIAC
D3	CIM disk monolithic purification principle	1	7	7	BIASEP
D4	REPP recombinant proteins, manual scale (10)	1	4	7	ADRIAC
D5	Not implemented REPP construction computer software program (REP-CSP)	1→3	18	26	ADRIAC HECUS
D6	Assessment tables for REPP antimicrobial activity (10)	2	40	9	UTS-BC UBIMMI
D7	2 x custom devices for the use of 96 well CIM® disks *renamed:* Thermo custom device for general use	3	14	26	HECUS
D8	CIM disk monolithic purification principle semi-automatic	1	12	12	ADRIAC BIASEP
D9	Short-list active REPPs	2	12	12	UTS-BC UBIMMI
D10	REPP recombinant protein expression construct library (semiautomatic-scale)	1	32	27	ADRIAC
D11	20 x CIM® disks specific for REPP purification in the 96 well format	3	30	26	BIASEP
D12	Not implemented Computer software, installation and documentation	3	27	26	HECUS
D13	REPP recombinant protein library (semiautom-scale)	1	27	28	ADRIAC
D14	Initial assessment tables for REPP libraries	2	68	28	UTSBC UBMMI
D15	Efficacy/cytotoxicity data table	2	14	30	UTSBC
D16	Exploitation Assessment Report and dissemination Plan	4	22	30	ADRIAC
D17	Exploitation Report Digest Hard and Web copies	4	1	31	ADRIAC

Table 2: Milestones based on a seven month project extension

		month	lead contractor
M1	Completion of 10 RE constructs with different PPs	6	ADRIAC
M2	Functional assembly CIM discs in 96 well format	8	BIASEP
M3	Functional 96 well purification device	15	HECUS
M4	Decision on REPP suitable for library design	26	ADRIAC UT-BC UBIMMI
M5	Decision on REPP class for extensive characterization	12	ADRIAC UT-BC UBIMMI
M6	480 construct REPP library	27	ADRIAC
M7	Functional semi automatic REPP isolation system	31	ADRIAC
M8	Project assessment and bench-marking meetings	31	ADRIAC UT-BC

Table 3: Financial Resources List

Contract N°:	**COOP-CT-2005-018191**			
PARTI-CIPANTS	**TYPE of EXPENDITURE (as defined by participants)**	**financial resources (EUR) Reporting Period 1**	**financial resources (EUR) Reporting Period 2**	**financial resources (EUR) Project**
Part. 1…….	**Total Person-month**	53	66	**119**
ADRIAC (SME)	Personnel costs	86706	101154	187860
	Major cost item 'equipment' *	0	0	0
	Major cost item 'subcontract' **	0	0	0
	Travel	4357	5643	10000
	Other costs ('the rest')	42028	50452	92480
	Total Costs	**133091**	**157249**	**290340**
Part. 2…….	**Total Person-month**	17	31	**48**
BIASEP (SME)	Personnel costs	33800	34200	68000
	Travel	2000	2000	4000
	Other costs ('the rest')	31600	16400	48000
	Total Costs	**67400**	**52600**	**120000**
Part. 3…….	**Total Person-month**	12	42	**54**
HECUS (SME)	Personnel costs	52393	40876	93269
	Travel	2528	2437	4965
	Other costs ('the rest')	28959	22807	51766
	Total Costs	**83880**	**66120**	**150000**
Part. 4…….	**Total Person-month**	44	38	**82**
UTSBC (RTD)	Personnel costs	64696	60404	125100
	Major cost item 'MS nano HPLC'	35000	13000	48000
	Travel	2248	7262	9510
	Other costs ('the rest')	50875	58235	109110
	Total Costs	**152819**	**138901**	**291720**
Part. 5…….	**Total Person-month**	23	33	**56**
UBIMMI (RTD)	Personnel costs	54967	65033	120000
	Major cost item 'equipment'	19805	3195	23000
	Travel	5000	5000	10000
	Other costs ('the rest')	34296	44304	78600
	Total Costs	**114068**	**117532**	**231600**
TOTAL	**Total Person-month**	**149**	**210**	**359**
	Personnel costs	292562	301667	594229
	Major cost item 'equipment'	54805	16195	71000
	Tavel	16133	22342	38475
	Other costs ('the rest')	187758	192198	379956
	Total Costs	**551258**	**532402**	**1083660**

Table 4: Description of Resource use by Workpackage

		WP1	WP2	WP3	WP4	Total
ADRIAC	**Total Person-month**	80	6	7	20	113
	Personnel costs	133.000	9.900	11.700	33.260	187.860
	Major cost items	0	0	0	0	0
	Travel	3.000	0	3.150	3.850	10.000
	Other costs ('the rest')	75.000	5.000	5.480	7.000	92.480
	Total Costs	211.000	14.900	20.330	44.110	290.340
BIASEP	**Total Person-month**	9	1	30	8	48
	Personnel costs	14.500	0	45.000	8.500	68.000
	Major cost items	0	0	0	0	0
	Travel	1.000	0	3.000	0	4.000
	Other costs ('the rest')	11.400	0	35.000	1.600	48.000
	Total Costs	26.900	0	83.000	10.100	120.000
HECUS	**Total Person-month**	10	2	35	7	54
	Personnel costs	17.488	1.943	58.293	15.545	93.269
	Major cost items	0	0	0	0	0
	Travel	789	0	2.226	1.950	4.965
	Other costs ('the rest')	9.706	1.078	32.354	8.628	51.766
	Total Costs	27.983	3.022	92.873	26.123	150.000
UTSBC	**Total Person-month**	12	70	0	2	84
	Personnel costs	17.000	105.100	0	3.000	125.100
	Major cost items	28.000	20.000	0	0	48.000
	Travel	0	8.000	0	1.510	9.510
	Other costs ('the rest')	10.000	97.000	0	2.110	109.110
	Total Costs	53.000	232.100	0	6.620	291.720
UBIMMI	**Total Person-month**	2	54	0	0	56
	Personnel costs	10.000	110.000	0	0	120.000
	Major cost items	7.000	16.000	0	0	23.000
	Travel	1.000	9.000	0	0	10.000
	Other costs ('the rest')	4.000	74.600	0	0	78.600
	Total Costs	19.000	212.600	0	0	231.600

Section 3. Consortium and Project management

3.1 Consortium management

Management tasks

The management was involved

- at the start of the project principally in ensuring rapid implementation of a consortium agreement and implementation of the project Web page to publicise the project;
- throughout the duration of the project in promoting and coordinating meetings amongst partners to ascertain compliance of partners with their tasks within each WP of the project,
- at the end of the project in obtaining information from all partners for the final report.

These activities were facilitated by the size of the project (five partners) and the favourable geographical distribution of groups, most of which were located in the Alpe-Adria region. This facilitated bilateral and WP meetings, which included visits by ADRIAC and UTSBC personnel to BIASEP in Ljubljana throughout the 1st year of the project, in August, September and December 2005 and April 2006, and meetings between ADRIAC and HECUS in Graz or Trieste in November 2005, February 2006 and May 2006. A WP2 meeting was held between ADRIAC, UTSBC and UBIMMI in Trieste on June 2006 to discuss intra-laboratory variations in REPP assay results, in Bonn in March 2007, to discuss bacterial internalisation problems, and in March 2008 in Lucca, at an international conference on antibiotics, to draw conclusions.

Communication between partners

Comunication in the ET-PA consortium was quite satisfactory and fruitful. In fact this turned out to be a strong asset of the project. Partners were encouraged to explore the possibility of co-operation also with other projects, and in particular EU ones within Framework 6 and 7. In this respect, components of the UTSBC and UBIMMI groups collaborated with Russian groups on an INTAS project on antimicrobial peptides which could be interesting bacterial penetration sequences, and are currently collaborating in a Marie-Curie People Industry-Academia project on novel antimicrobials. ADRIAC and UTS-BC are collaborating in several Friuli-Venezia-Giulia projects on development of novel anti-infective agents and continued REPP development. The UTSBC partner is also taking part in an EU integrated project for SMEs entitled "Nanobiopharmaceutics" aimed at developing novel delivery systems for drugs, and an ET-PA representative assisted at its kick-off meeting so as to obtain information on possible synergies.

Contractors

All partners were active from the beginning of the project, although the nature of the project required the ADRIAC, BIASEP and UTSBC to be the most active in this phase of the project. No changes in responsibilities were required nor were any changes made to the consortium itself. ADRIAC decided not to activate a subcontract for custom DNA construction software, due to adjustments in objectives, as discussed in Section 1, *Main problems and corrective actions undertaken*, Problem 5, and in Section 2, *Deviations from the project work programme and corrective actions taken*. Funds allocated for this will thus not be requested

Coordination activities.

To avoid a potential bottleneck at the beginning of the project, it was necessary to rapidly provide a REPP biomolecule prototype to the RTD partners so that they could start setting up REPP-specific assays and validate specific methodologies. Later, problems listed in Section 1 had to be promptly faced and solved. The fact that the project coordinator was also technically involved in all WPs increased problem-solving capacities of the consortium. The coordinator always had the support of all partners in preparing reports and deliverables, and their collaboration with required meetings.

3.2 Project management

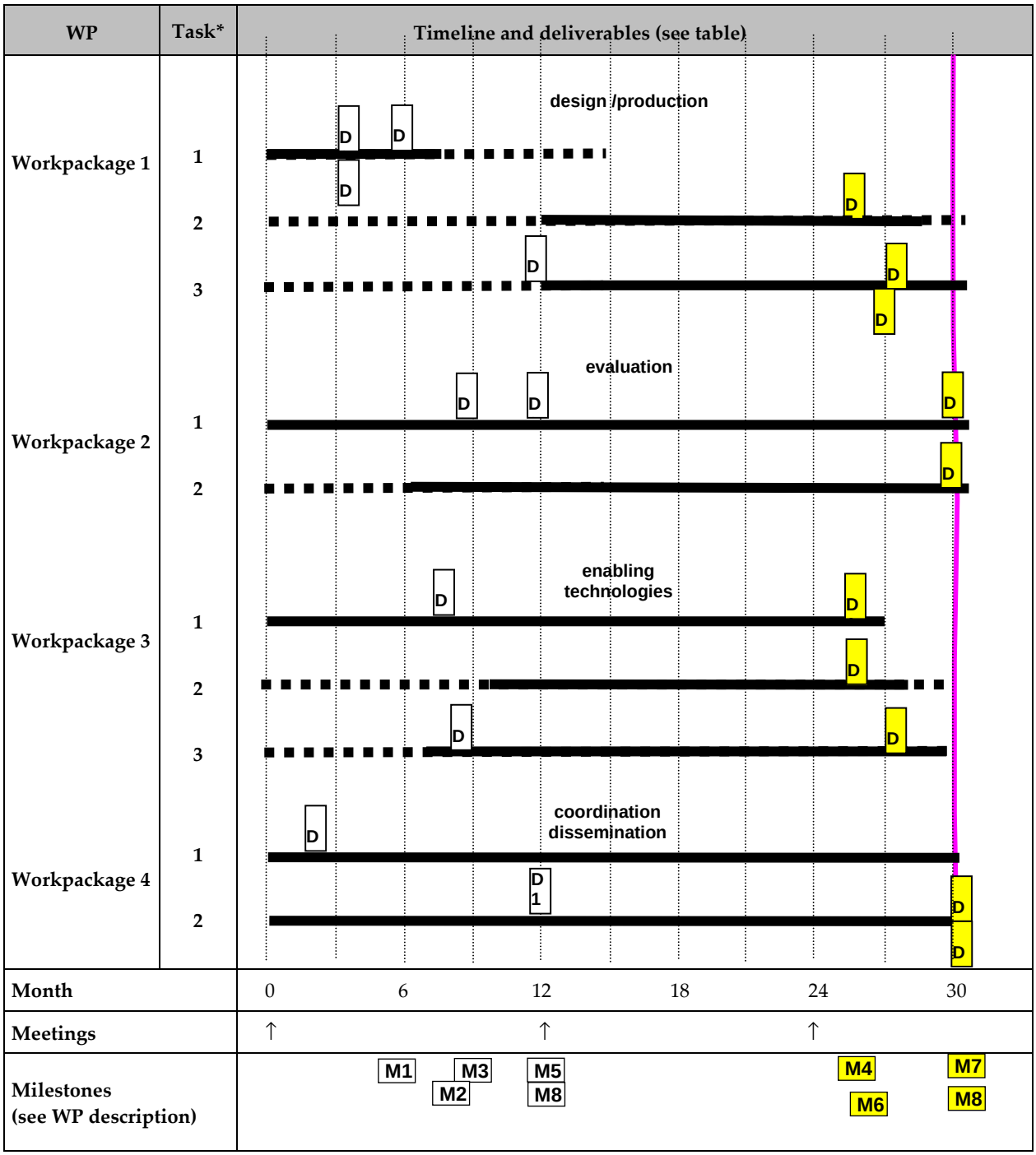
The management involved

- at the start of the project principally in ensuring rapid start to collaborations between partners;
- throughout the duration of the project in ensuring each partners complied with the assigned tasks
- solving scientific technical problems as they arose,
- monitoring compliance with the project timetable and if necessary implementing rescheduling
- at the end of the project in obtaining technical information from all partners for preparation of deliverable technical sheets .

These activities were facilitated by the fact that all partners entered easily into their assigned collaborations and communicated adequately. It was also helped by a climate of mutual trust which allowed a certain informality in Partner interactions which made collaborations more efficient.

Project timetable

Workpackages - Plan and Status Barchart



The **research tasks** of each participant group have been organised into three technological work-packages (WP1-3) and into one coordination/dissemination work-package (WP4) as summarized:

WP1) **Design, production and REPP protein purification technology;**

WP2) **REPP biological activity evaluation and optimisation**;

WP3) **Purification, semi automatic REPP isolation system and embedding technology;**

WP4) **Consolidation, intellectual property and end user management;**

Changes and impact on milestones.

The project timetable required some reassessment and changes to take into account problems and solutions listed in Section 1. In particular, delay was experienced in deciding on the short-list of active REPPs to further develop, due to the factors discussed in Section 1 (see *Main problems and corrective actions undertaken)*.

Milestones 1-3 are on track.

Milestones 4 and 5 are now partly met regarding antibiotic action, in that the potentially most useful PP sequences for internalization into some types of bacteria have been identified, but one family is not yet fully characterised.

The delay in finalising M4 and 5 obviously affect Milestone 6 (480 construct REPP library). As all problems relating to REPP folding, stability and DNAse activity were effectively solved during the ET-PA project, and CIM purification technology proved entirely successful, the possibility to construct up to a 480 REPP library is now entirely feasible. Current REPP production capacity is over 10 new constructs per week. This milestone will not however be implemented until a final decision on the REPP family or families for future development. Progress made during ET-PA has lead to an enormous boost of the parallel anti-tumor programme, so it is likely that an anti-tumor REPP library will be developed in parallel.

Regarding milestone 7 (Functional semi automatic REPP isolation system), this has been met, modifications were agreed for termo device key priorities towards a more directly marketable product.

Regarding milestone 8 (Project assessment and bench-marking meetings), an extensive project reassessment has been carried out after P1 (month 14) and in several meetings during P2 (see section 3 Management tasks).

Section 4 Ethical issues

Animal studies were performed in accordance with EU and the National Institutes of Health guidelines. These experiments were carried out exclusively in ITALY at the animal house facility of the University of Trieste, in Trieste, Italy, which has obtained approval to conduct its activities from the italian Ministero della Sanita, Dipartimento degli Alimenti e Nutrizione e della Sanita Pubblica Veterinaria, Decree No 115/2000-A. The regulations governing animal experiments, as set out in the national law (116/1992) were strictly adhered to by Adriacell researchers acting in collaboration with the qualified staff of the animal house. The national regulations governing the functioning of this facility are available at its web site: http://www.univ.trieste.it/~servpoli/dl116a92.htm. It is further noted that this national law complies with the EU legislation 89/609/CEE.

In vivo experiments involving animals were carried out after extensive *in vitro* characterization of the antimicrobial activity of the REPP constructs, and after evaluation of their cytotoxic activity in well established cell culture assays. The use of living animals was kept to a minimum, and it was decided not to carry out toxicity studies within the ET-PA project as these were considered premature. *In vivo* experiments in mouse models were carried out only to test the bioavailability and stability of selected optimized constructs. The minimum number of animals was used to allow determination of the serum half life and residual enzymatic activity of two REPP constructs. This was considered to be essential to confirm the optimisation process. Permission for these experiments was requested in Q3/2006 to the italian Ministero della Sanita, and subsequent to approval by the Istituto Superiore della Sanità of the submitted protocols, was duly received. Experiments were carried out according to the national rules governing this type of testing. The two experiments required 5 Balb/c mice for the control REPP construct and 10 mice for the optimized REPP construct. Single bolus injections at the subtoxic level of a 3mg/kg were used in each experiment. At given times the animals were sacrificed to recover samples.

Work including Human beings or tissues requiring informed consent was not contemplated by the project

Micro-organisms employed either as cloning or expression hosts for semi-large scale production fell under the category of S1 organisms, which is characterised by European legislation as safe, *i.e.* there are no reported cases of infections of humans. Genetic manipulations were conducted under the regulations applying to the S1 group. The production strains have the GRAS (generally-recognised-as-safe) status. None of the GMOs created or used throughout the work was released to the environment. Genetically modified organisms used for REPP construct production had *E.coli* XL1 or XL10 blue MRF' background only.

Pathogenic micro-organisms were used by partners UTSBC and UBIMMI in efficacy assays. These pathogenic micro-organisms fall into category S2. Both partners made use of Laboratories of Safety Standard L2 and L3 (according to standards released by the EU).

Qualification of persons involved in the experiments. All the work was carried out exclusively by personnel that is specifically trained and instructed to carry out such experiments. Waste material from such work was routinely sterilised before being disposed of. Animal carcasses, deriving from the animal model studies described above, were chemically sterilised or autoclaved before being disposed of as biological waste.

Section 5 Other issues

Overall contributions of each contractor

Contribution of the ADRIAC partner

- design, vector construction expression and purification of REPP biomolecules (WP1)
- testing CIM disc technology for application in REPP biomolecule production (WP1)
- development of devices based on 96 well CIM technology (WP3)
- development of integrated process control software (WP3)
- management (WP4)

ADRIAC collaborated with RTD partners in the selection of penetration peptide (PP) classes suitable for REPP internalisation into pathogenic micro-organisms. Using a short-list of over 100 different PP candidates, taken from numerous AMP sequences as well as the majority of reported CPPs for ekaryotic cells and organelles, over 150 REPPs were assembled as recombinant REPP expression vectors. The expression system was completely optimized and the proteins were then expressed and subjected to purification. Major optimisation steps for the entire molecular REPP design, requiring codon optimisation and asymmetric codon design for the two individual subunits of the PvuII homo-dimer, construction of improved PvuII expression vectors with flexible poly-linker design at the N-terminus, structure based engineering of an optimal subunit-linker; optimizing the production strain for REPP proteins has reduced the time from design to expression from several months to a few days. Design changes aimed to protect from extracellular, constitutive or stress induced proteases and serum sequestration has increased in-vivo lifetimes from minutes to days.

One key step in the REPP gene assembly process is semi-automated oligonucleotide purification, for which CIM disc technology into 96 multi-well formats (96CIM) will be required . 96 well CIM disk plate prototypes were successfully completed by BIASEP and these were tested by ADRIAC. ADRIAC also collaborated with HECUS on the development of a semiautomatic thermo "iso-vapor" device for using the 96-well multi-well CIM format. With HECUS, it also defined the elaborated techniques required for semi-automated REPP vector construction, protein expression and purification.

Contribution of the BIASEP partner

- delivery of CIM disks of various chemistry for manual handling (WP1)
- co-developing chromatographic methods for implementation of CIM technology (WP1)
- development of 96-well plates containing CIM monoliths (WP1)
- scale-up of CIM technology for larger scale production.

CIM monolithic disk can be implemented for different molecules applying different interaction modes. Because of that BIASEP has supplied to partners ADRIAC and UTSBC CIM disks of various different chemistries so as to find optimal solutions for particular systems. In this respect, separation of peptides used reverse phase (UTSBC) whereas that of oligonucleotides used metal chelate chromatography or ion-exchange chromatography (ADRIAC). In particular we have together with ADRIAC investigated the possibility for separation of long oligonucleotides from shorter incorrect sequences. This system was in fact found to be very complex, therefore a comprehensive study of oligonucleotide-matrix interactions was performed. It turned out that despite the fact that the number of binding sites increases with oligonucleotide length, the elution ionic strength becomes independent of the length and therefore poor separation takes place. Different strategies are currently investigated to overcome this problIn parallel, prototypes of 96-well plates using CIM monolithic discs were prepared. Two main strategies were investigated: inserting pre-polymerised monolithic disks into wells, or polymerising monoliths directly in the 96-well plate. Advantages of each approach depend on the 96-well plate type, as well as of the expected application. Both approaches resulted in uniform flow through the wells, exhibiting similar flow rates at given pressure difference. Several tests for oligonucleotide separation using the prepared 96-well plates containing CIM monoliths have already been performed by ADRIAC.

Contribution of HECUS partner

- To develop process control software for REPP vector construction, protein expression and quality control for final implementation into the process controlled technological platform
- Development of required hardware that is not commercially available.

HECUS has worked with ADRIAC on the definition of flow diagram that describes the elaborated techniques for REPP vector construction, protein expression and purification from WP1 that will serve as the working scaffold in period 2 of the ET-PA project (P2).

HECUS has also worked on the design and assembly of key hardware which is required in the REPP production process for translation of the manual scale principles into a semi-automatic process, and that is not commercially available. One key step was to develop a device for using the 96 multi-well formats CIM disc technology developed by BIASEP to enable its use in a semiautomatic mode. A thermo "iso-vapor" device is being constructed that facilitates the temperature-controlled use of such 96 CIM multi well disks. The temperature regulation needs long holding periods (up to 12 hours) with linear temperature changes at variable time intervals, and HECUS has implemented temperature programs compatible with these long periods but also with the humidity control required of the minimal volumes used (column volume + 10-20%, 40 to 60 µl for 96 format). The device also takes into consideration the necessity to directly cover the CIM devices providing direct contact so that the necessary temperature gradients are obtained. A control unit with chamber and thermo-blocks is now being assembled.

HECUS also designed, implemented and maintains the project web-site.

Contribution of UTS-BC RTD partner

- Re-assesment of PP for bacterial penetration
- Development of more sensitive assays
- Semi synthetic RE-Cys-Cys-PP constructs

Regarding the activities of the UTS-BC group to meet its objectives, all tasks proceeded well without any significant problems. In this respect, an extensive scrutiny of available data and the literature was carried out initially with the UNIBONN partner, and resulted in a list of 35 potential penetration peptides provided to WP1, which added it to its PP database. REPP constructs were tested for antimicrobial activity by UTS-BC in collaboration with UNIBONN. As part of its efforts to characterize penetration peptides for WP1, UTS-BC has also synthesized 10 potential penetration peptides of different types modified with a c-terminal cysteine. This allows both fluorescent labelling to allow the study of bacterial cell penetration by flow cytometry, and linking to Cys-modified RE to produce REPP constructs in an alternative manner. Extensive SAR studies were carried out on selected types of penetration peptides (e.g. cytolytic LL-37, translocating Bac-7) using biophysical and biochemical methodologies.

UTS-BC has also identified a component of the putative transport system for Pro-rich antimicrobial peptides (AMPs) into some Gram-negative bacteria. The Pro-rich peptides produced by some mammals can have considerable sizes (up to 100 residues), but only relatively short N-terminal sequences are necessary for transport, so that they could act as effective PP for protein cargo. The transport system for Pro-rich AMPs was recognized by a genetic method based on identification of bacterial genes that, if modified, result in decreased AMP susceptibility. Amongst these was the *sbmA* gene coding for a transmembrane protein involved in the uptake of various peptides. This protein was found to be the transmembrane domain of a

putative ABC transport system, and it has been shown it to be involved in translocation of several different types of Pro-rich peptides. In particular, by using such AMPs labelled with the fluorescent molecule BODIPY, it was conclusively shown that these were internalised without membrane lysis. Some of the obtained data, which is amenable for public disclosure, has been published (see references) as part of the dissemination activities. UTS-BC has developed a protocol for preparing semi-synthetic RE-Cys-Cys-PP constructs by covalently binding a chemically synthesized Pro-rich peptide to an expressed RE module, via disulfide bond formation. This is intended to overcome expression problems met by ADRIAC in producing REPP containing Pro-rich PP (see *Main problems and corrective actives taken section*).

The UTSBC invested time in developing alternative, more sensitive antimicrobial activity assays, essential for future analysis of the larger numbers of REPP. A *Pseudomonas aeruginosa* strain was obtained that expresses the luxCDABE (luciferase) gene cassette, grows normally and expresses luminescence in a constitutive fashion. This has initially been tested using numerous sequences chosen from among the PP considered for REPP construction and while at high PP concentrations (several times the MIC), the expected decrease in luminescence was observed, at lower concentrations (at or below the MIC), an initial strong increase in fluorescence was observed. This likely derives from the bacterial cell reacting to antibiotic offence by ramping up parts of its metabolism. Work is ongoing to better understand these aspects, and also to determine the most stable assay conditions for maximum reproducibility. Once the assays are completely defined, the advantage is that by performing them at sublethal REPP concentrations, information can be gathered on bacterial reaction to offence, and help develop methods to overcome these, thus helping prevent resistance development.

The UNI-BC group has worked in close and constant collaboration with the ADRIAC SME partner, which has also involved the exchange of personnel. It is also worked to set up alternative optimised peptide-purification protocols with the BIASEP SME partner, involving its proprietary CIMM disk monolith technology. UTS-BC is acting in WP4 to promote dissemination of results by publishing, or otherwise making publicly available data deriving from the project which is cleared as not sensitive by SME partners. It also actively collaborated in the creation and maintenance of the project web page. In conclusion, the UTS group has worked to meet all its objectives for the reporting period and has co-ordinated extensively with other groups that compose the consortium, regarding the flow of information, material and personnel.

Contribution of the UBIMMI group

- REPP assessment
- REPP testing on intracellular pathogen

The Bonn group is primarily involved in WP2 in which the main tasks are the characterization of activity and the evaluation of efficacy. For testing the clinical relevance of a given restriction enzyme penetration

peptide (REPP) construct, standard laboratory strains such as *M. luteus* are of little use. The Bonn group owns a set of about 300 fresh clinical isolates with well characterized antibiotic resistance profiles and covering the most problematic species and genera with regard to resistance development. For the initial screening of incoming REPPs we compiled a set of four representative multiresistant strains (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Enterococcus faecium*). In addition, we used the standard laboratory strain *M. luteus*, which is sensitive to a large range of antimicrobial peptides. The activity testing was done in standardized broth microdilution assays using polypropylene microtiter plates which are supposed to minimize binding of molecules with hydrophobic moieties. All REPPs received from partner 1 were tested, however, MIC determinations did not reveal relevant activities against several clinical isolates, but some activity against *M. luteus* and the *S. aureus* 8325 strain. Test for membrane depolarisation indicated that in the presence of the active REPPs, substantial depolaristaion was not observed, indicating that the cidal activity did not derive from a lytic activity of the PP portion.

In order to enhance the activity of the REPPs, partner 1 modified one of the peptides which indeed resulted in higher activity for ATCC laboratory strains but not for the clinical strains. REPPs have also been tested against the intracellular pathogen *Mycoplasma pneumoniae* so far unsuccessfully, but the method is now set up for testing new generation REPPs.

Furthermore, pulsed field gel electrophoresis (PFGE) was performed, which is a method using an electrical field with alternating polarity for separation of extremely large DNA fragments. The PFGE results in a fingerprint of large chromosomal DNA fragments produced by restriction enzymes and the idea was to detect visible changes in the restriction pattern of a given strain after application of a REPP. Use of PFGE with the susceptible *M. luteus*, using this resulted in an excessive number of restriction sites for the enzyme in use (Sma1) to be useful. A useable PFGE system has instead been installed for the use on *S. aureus*, which will shortly be used to test the efficacy of the restriction activity in this bacterium.

Some of the more promising REPP constructs were based on a small peptide called PepI, which is a 69-amino-acid immunity peptide encoded by the Pep5 biosynthesis gene cluster in order to provide producer self-protection. Pep5 is a cationic pore-forming lantibiotic produced by *Staphylococcus epidermidis* 5, which is active against gram-positive bacteria. The immunity peptide PepI is characterized by a hydrophobic N-terminal segment and a strongly hydrophilic C-terminal part with a net positive charge. PepI fused to green fluorescent protein (GFP) was found to translocate to the outside of the bacterial cell, and to accumulate at the membrane-cell wall interface. GFP is a 27-kDa protein and by fusion to PepI, the 69-amino-acid immunity peptide turned into a 35-kDa fusion protein, and it was most remarkable to find that a fusion protein of this size was directed across the membrane with the same efficiency observed for the small native immunity peptide. Therefore, it was suggested that the N-terminal part of PepI may be used as sort of a vehicle to transport the REPPs into the cell. The target of the REPPs is the chromosomal DNA inside the cells and one of the major problems is to make sure that the REPPs reach their target to perform their lethal

activity. During these experiments, an *S. carnosus* strain also expressing and exporting PepI fused to GFP has been developed. This was provided to UTS-BC for the development of antimicrobial assays based on fluorescence inhibition, which could be more sensitive than reduced absorption due to scattering.

Annex 1

Plan for using and disseminating knowledge

Section 1 - Exploitable knowledge and its use

Exploitable result	Exploitable product(s)	Sector(s) of application	Market entry	IPR protection	Owner/s
REPP biomolecules	*REPP based antibiotics*	*1. Biomedical*	*2014*	*Product follow up patent family IP 1616011*	***ADRIAC***
3. REPP biomolecules	*REPP based antitumorals*	*1. Biomedical*	*2012*	*Product follow up patent family IP 1616011*	***ADRIAC***
96 well CIM disks	*CIM technology in multiwell format*	*1. Diagnostics 2. Proteomics 3.Molec.biol.*	*On the market*	*Added value to existing patent family*	***BIASEP***
5. Thermo device	*Hardware/software/applications*	*1. Bioproduction 2.Nanotechnology*	*On the market*	*Under consideration*	***HECUS***

Description of exploitable results:

1&2. *REPP biomolecules* - Potential new class of antibiotics and antitumor lead substances

3. *96 well CIM disk* - Integration of CIM technology into multi-well format represents a catalogue product for BIASEP, having applications for high-throughput peptide, protein or oligonucleotide purification.

4 *Thermo device* - Versatile hardware/software/applications package for use with SAX/WAX instrument allowing monitoring, batch-control and quality-management of pharmacological compositions, including biopharmaceuticals.

Section 2 - Dissemination of knowledge

Overview table

Dates	Type	Type of audience	Where	Audience size	Partners involved
12/05	*1. Project web-site*	*General public*	*International*	*large*	*ADRIAC UTS-BC*
05/06	*2.. Conference*	*Research*	*International*	*150*	*BIASEP*
05/06	*3. Flyers*	*General Public*	*International*	*large*	*ADRIAC*
10/07	*4. Publication*	*Research*	*International*	*large*	*UTS-BC*
12/07	*5. Publication*	*Research*	*International*	*large*	*UTS-BC*

Description of activities

1. Project web-site - The project web-site was set up for internal use early on in the project, and after consolidation and suggestions by partners, a public version was ready by early 2006. The site has been continuously updated and contains information on the ET-PA REPP concept for public dissemination, information and links to the SME and RTD partners, other useful links pertaining to the project, and the schedule for ET-PA project meetings, as well as photo records of these meetings which are accessible to the general public. Presentations made during the meetings are accessible only to ET-PA members in a restricted area, where other sensitive information can be posted. The web page was set up in a format which allows for considerable flexibility in web page modification and updating by the different project partners through a restricted administration function.

2. Publications - Information on REPP design, construction and activities is premature for publication, However, data gathered by the RTD groups on the mode of action of potential penetration peptides and methods to study internalisation or membrane interactions has been cleared by the SME partners for publication in international refereed scientific journal. The ET-PA project was acknowledged.

1) E. Podda, M. Benincasa, S. Pacor, F. Micali; M. Mattiuzzo, R. Gennaro, M. Scocchi "Dual mode of action of Bac7, a proline-rich antibacterial peptide" Bicochem. Biophys. Res. Commun. 2006 1760:1732-1740

2) Role of the Escherichia coli SbmA in the antimicrobial activity of proline-rich peptides. Mattiuzzo M, Bandiera A, Gennaro R, Benincasa M, Pacor S, Antcheva N, Scocchi M. Mol Microbiol. 2007 66:151-63

3) Scocchi M., Mattiuzzo M., Benincasa M., Antcheva N., Tossi A., and Gennaro R. (2008) Investigating the Mode of Action of Proline-rich Antimicrobial Peptides Using a Genetic Approach: a Tool to Identify New Bacterial Targets Amenable to the Design of Novel Antibiotics. In Peptide-based Drug Design - Methods in Molecular Biology Vol. 28, L. Otvos Ed., Humana Press (in press)

4) F. Morgera, L. Vaccari, L. Creatti, N. Antcheva, A. Tossi (2008) "Structure and activities of AMPs at the bacterial membrane" in Membrane-active peptides: methods and results on structure and function. M. Castanho Ed. IUL Publishers (International University Line), La Jolla, California, USA (in press).

5) F. Morgera L. Vaccari, N. Antcheva, S. Pacor, A.Tossi (2008) "Primate cathelicidinsorthologues display different structures and membrane interactions" Biochem. J (in press)

6) A. Tossi, F. Morgera N. Antcheva, D. Scaini, S. Pacor, L. Vaccari, (2009) "Membrane interactions of primate antimicrobial cathelicidins LL-37 and RL-37" Proceedings of the 30th European Peptide Symposium, Helsinki, 2008 (in press)

7) R. Gennaro, M. Benincasa, S. Pacor, G. Carlini, A. Tossi, M. Scocchi (2009) A flow cytometric method to detect internalization of antimicrobial flourescently labelled peptides into bacterial cells. Proceedings of the 30th European Peptide Symposium, Helsinki, 2008 (in press)

ADRIAC – *Adriacell,* C. Kuhne Ricercainnovazione Meeting, Padova April 2008
www.ricercainnformazione.it

ADRIAC – *Adriacell,* C. Kuhne Corrinna Meeting – Promoting crossborder research and development cooperation, Vienna November 2007

UTS-BC – *Uptake of Bac7, a proline-rich antibacterial peptide, into the E. coli cells*, Maura Mattiuzzo, Monica Benincasa, Marco Scocchi, Sabrina Pacor, Antonella Bandiera, Nikolinka Antchevaa, Renato Gennaroa 2nd workshop on biophysics of membrane-active peptides Lisbon 2007

UTS-BC *A flow cytometric method to detect internalization of antimicrobial peptides in Gram-negative bacterial cells*, Monica Benincasa, Pacor Sabrina, Pelillo Chiara, Gennaro Renato, Scocchi Marco 11th Naples Workshop on Bioactive Peptides, Naples 2008

UTS-BC *Membrane interactions of the human antimicrobial cathelicidin LL37*. Francesca Morgera, Lisa Vaccari, Nikolinka Antcheva, Sabrina Pacor and Alessandro Tossi
11th Naples Workshop on Bioactive Peptides, Naples 2008

Conference Organisation - *BIASEP* has organised the 2008 and 2006 Summer Schools on Monolith Technology for Biochromatography, Bioconversion, and Solid Phase Synthesis at the end of May in Portorož, Slovenia. This provided attendees the opportunity to learn from a group of international experts about basic knowledge, research trends, method optimization, trouble shooting, and the integration of monoliths into already existing R&D, Production, or QA processes.

http://www.biaseparations.com

Flyers - All SMEs have produced flyers describing their activities and products which are available in hard or pdf formats.

Section 3 - Publishable results

Publishable results on the REPP concept will not be available until intellectual property is sufficiently protected. Cleared general information on the REPP concept is provided on the web page.

Data gathered by the RTD groups on the mode of action of potential penetration peptides and methods to study their internalisation or membrane interactions has been cleared by the SME partners for publication in international refereed scientific journal, and a publications list is given in the previous section on Dissemination. The ET-PA project was acknowledged.