It is well established that the normal microbial flora (microbiota) has a pivotal role in the biological homeostasis of the host. Elimination or reduction of the microbiota may result in harmful immunological and metabolic consequences, furthermore opens a niche for pathogens to colonize the involved area and induce infections. Probiotics are widely used to counterbalance the effects adversely influencing the microbiota. It has been noticed that persons with asymptomatic bacteriuria (ABU) much less frequently develop recurrent urinary tract infections (UTI) than the average population. This observation has lead to the idea that urinary isolates from such persons, to the analogy of probiotic bacteria, might be utilised in the prevention of recurrent UTI. In 2010 we joined an international consortium to evaluate this possibility.

To satisfy all the conditions required from a so called "bacterial interference" strain it has to meet not only efficacy but also safety requirements. Safety is an absolute prerequisite for ABU strains to be used for interference. The following aspects of the candidate interference strains have been investigated:

1. Genetic background and possible clonality.
2. Phenotypic appearance of virulence traits.
3. The calibre to acquire virulence and/or resistance genes when incidentally communicating with other, especially pathogenic bacteria.
4. The capacity to outcompete UTI strains in human urine and in an ascendent UTI mouse model.
5. The chance to cause symptomatic infection if incidentally reaching the circulation.
6. The ability to activate the host's immune system to produce inflammatory response.
7. To elicit the production of harmful substances by interference with the host's metabolism.

Accomplishments

1. We investigated 10 asymptomatic bacteriuria (ABU) *Escherichia coli* strains for the following virulence genes by PCR: ***hlyA***: α-haemolysin structural gene, ***hlyF***: haemolysin F, ***sheA***: silent haemolysin ***papC***: P-fimbrial outer membrane protein, ***papE/F***: P-fimbrial minor subunits, ***papGI***, ***papGII***, ***papGIII***: P-fimbrial adhesin subunits, ***fimH***: type-1 fimbrial subunit, ***sfaC/D***: S-fimbrial subunits, ***iucD***: aerobactin. Out of the investigated set of isolates *fimH* was present in all of the isolates. Variuos ***papG*** determinats were found in 4 strains, and S-fimbria genes in 5 strains. From the haemolysin genes *hlyA* was the most frequent being represented in 5 isolates. *iucD* for aerobactin was found in 4 strains.
Pulsed field gel electrophoresis patterns of the ABU isolates were diverse. They belong to different *Escherichia coli* phylogenetic groups (A, B2, D).
One ABU *E. coli* genome has been fully sequenced by the German consortial partner and sequencing of one of our isolates is in progress here in Hungary. Completing, evaluation and comparative annotations will be performed in the near future

2. Contrary to the genotypic frequency of virulence traits their appearance in the phenotype is much less frequent. Only one strain expressed functional type-1 fimbria, and none of them P- or S-fimbriae. Four strains elicited haemolysis on blood agar, and five strains produced aerobactin. Further virulence phenotypes, which can be of multigenic functions were also tested. These include cell adherence (5 strains positive), cytotoxicity (2 strains positive), serum resistance (6 strains positive) and biofilm production (4 strains positive). However, in adhesive and invasive capacities the "positive" ABU strains did not reach the values observed with the reference UTI strains.

Our finding that only one strain expressed a single fimbria species though the presence of fimbria genes was much more frequent partly support the assumption that ABU strains emerge from UTI strains by degeneration of the functionality of virulence genes. On the other hand 4 strains did not harbour haemolysin, P or S fimbria genes at all which are the most specific and characteristic UTI genes. This finding at least makes questionable the general validity of the "degeneration" assumption. Furthermore, phylogenetic typing revealed that ABU strains may belong to groups of both extraintestinal pathogens (B2 and D) or to commensals (group A). The former might have „developed" from UTI to ABU strains by degeneration but the latter must have been indigenously non pathogenic.

3. Inactivated virulence genes in ABU strains may develop in two ways. Either pathogenicity islands with active virulence genes are taken up, and later suffer mutations (that is, first a pathogen develops and gene inactivations lead to the ABU character) or the genes are already not functional when taken up. By pathogenicity island transfer experiments we could show that a whole pathogenicity island may be transmitted from an UTI strain to an intestinal commensal strain. In some cases, however, only a part of the island was transferred. This shows that incomplete virulence gene sequences may also be transmitted, and ABU strains are not necessarily inactivated derivatives of uropathogenic strains. Beside in vitro conditions we could achieve the island transfer also in vivo in the mouse intestine. The frequency of transfer was in a 10^{-8} range.
To check the possibility of conjugal transfer of resistance determinants we tried to conjugally transfer a composite sequence of haemolysin and tetracycline determinants from a UTI strain into two selected ABU isolates not harbouring the above genes. In these trials we were not successful even when using as high cell concentrations as 10^{9}. In transfer experiments with a multiresistance plasmid we received also negative results. These studies suggest that with the highest bacterial counts conceivably present in a supposed encounter of ABU and UTI strains in the bladder transfer of resistance determinants is not likely to happen.

4. The outcompeting experiments were successful when a UTI strain was co-cultured with ABU strains in human urine both in our laboratory and in other laboratories of the consortium. At our hands the 1:1 ratio turned to 20:1 for the favour of the ABU strain within 24 hours of co-cultivation. However, the ascendant mouse UTI model was not suitable to evaluate this issue *in vivo* as ABU strains were eliminated even if they were introduced into the mouse bladder without coinfection with UTI strains.
Characterisation and outcompeting studies with multiresistant *Klebsiella* pathogens and ABU *E. coli* have been started.

5. The consortial partners in Sweden have performed ethically licensed human trials showing promising results for the interference capacity of an ABU strain against UTI isolates. Before recommending bacterial interference for more extended application it is mandatory to assess if an incidental translocation of the ABU strain into the surrounding tissues or even into the circulation can elicit infectious complications. This delicate issue could not be evaluated in human trials but only in mouse models. So the results can be extrapolated to human conditions with some reservation. It is clear that haemolytic ABU strains can never be applied for interference. Our non-haemolytic ABU isolates together with the Swedish interference strain produced no symptoms after intravenous infection in mice when an as high

dose as $5x10^8$ bacterial cells were injected intravenously. Beside the lack of the haemolytic character this might be due to the serum sensitivity of the strains.

6. UTI induces local and general immune responses including cytokine and antibody production, and also leukocytosis. We could show raises in *E. coli* anti-haemolysin titres in UTI patents but it was not the case in ABU persons when their ABU isolates were haemolysin producers. We also demonstrated increasing titres against surface antigens of the pathogens in UTI patients. ABU, inherently to its definition, is known to produce no subjective symptoms but mild leukocyturia may occur. The ABU persons presented with no cytokine response or raised antibody levels to the surface antigens of their isolates either. As some ABU strains showed a mild autoaggregating capacity, we performed lipopolysaccharide electrophoresis. Upon the results we suggest that ABU isolates might have degraded LPS. It fits with the serological analysis of the interference ABU strain by the Swedish partner. That strain has a rough LPS, produces no fimbria but harbours K5 capsular antigen. K5 antigen is desulfo-heparin which is an intermediate in heparin synthesis, and so it is not recognised as foreign. In our mouse models including intravenous infections with 5×10^8 cells this strain did not elicit any death or even any symptoms. So we assume that this strain with its immunologically inert surface, furthermore with no motility and haemolysin negativity seems to be the best candidate for further interference studies also from the point of view of safety. This assumption is further supported by our finding that the strain is killed in human serum within one hour.

7. ABU is a relatively frequent condition among diabetic patients. They use artificial sweeteners instead of sugar**.** The artificial sweetener cyclamate (cycloheximine sulfate) can be split by bacterial sulfatases to cycloheximine which is a bladder carcinogen. Diabetic patients are not always aware how many artificial sweeteners they consume as cyclamate is taken not only in pills but also with diabetic “light” beverages. The consumed cyclamate is excreted in unchanged form with the urine. So we think that for safety reasons it is absolutely important to know if candidate interference strains have the capacity to split cyclamate, and produce the carcinogen just on the spot, in the bladder. Therefore we started to establish an experimental model to investigate this question. At present we are at the step of standardisation of mass-spectrometric an HPLC determination of cyclamate and cycloheximine concentrations in order to measure the effect of ABU strains on cyclamate when cultured in human urine at the presence of cyclamate.

Up to now we have a publication with the first authorship from our group, and several conference abstracts. For the explanation of this moderate publication activity I give the background information that three researchers and the only technician involved in the project left our team during the project tenure for abroad, for permanent jobs offered in other institutes, or retired. So even the presented experimental work was partly done with the help of persons working basically on other projects. Further full publications are envisaged to come from the experiments being in progress.