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Probing of *Enterobacter cloacae* acid inducible *asr* gene in mouse model

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The *Escherichia coli asr* gene and its orthologues in the genomes of other members of the family *Enterobacteriaceae* represent stress response elements regulated by a low environmental acidity (pH < 5.0) [1]. They code for small basic 82–139 amino acid proteins of unknown function. While important for exponential phase *E. coli* growth in liquid culture at pH 4.5, *asr* is also required for bacterial colonization of the lower intestine of the animal host under competitive pressure and for competition in long-term culture [2]. Here we demonstrate that the *Enterobacter cloacae asr* gene is able to restore the colonization deficiency of the *E. coli asr* mutant under competition in the mouse model. *E. cloacae* Asr protein shows a 76% amino acid identity to *E. coli* Asr and undergoes proteolytic processing induced by acid stress. These observations imply that the *asr* gene function is conserved through *Enterobacteriaceae* and is important for animal host colonization.

Key words: *Enterobacter cloacae*, *asr* gene, mouse gastrointestinal tract colonisation

INTRODUCTION

The response of *Enterobacteriaceae* to changes in pH is an important aspect of bacterial survival in the natural environment, including the animal host. Many bacteria are killed by the low gastric pH (pH 1.0–2.0) [3, 4]. The oral-faecal lifestyle of *Escherichia coli* and other enterobacteria is facilitated by their ability to survive a low pH. Acidic pH leads to upregulation of a wide range of genes [5–7] which are proposed to be components of acid survival and resistance systems. These proteins function in biochemical reactions such as neutralizing of external acid, adjusting cellular catabolism to a new environment, performing DNA repair and membrane biogenesis, acting as chaperones, and contributing to the microbial pathogenesis [5–8], and are expected to act coordinately to provide an efficient protection against acid stress.

The *E. coli asr* gene (*a*cid *s*hock *R*NA) is strongly induced by a high environmental acidity (pH <5.0) [9]. The gene encodes a protein of unknown function, which is required for bacterial growth at a moderate acidity (pH 4.5) as well as for the induction of acid tolerance at a moderate acidity. Inactivation of *asr* results in an acid-sensitive phenotype [10]. *E. coli* Asr is a periplasmic protein synthesized as a precursor with an apparent molecular mass of 18 kDa. The Asr protein precursor undergoes N-terminal cleavage of the signal peptide and an additional cleavage via an unknown protease yielding 8-kDa polypeptide. The second processing event occurs most probably in the periplasm. Asr processing has been shown to be important for *E. coli* growth at acidic pH (pH 4.5) [10].

Homologues of the *asr* gene are found mostly in the genomes of the family *Enterobacteriaceae* members and have been shown to be induced under acid shock conditions [1, 10]. They code for 82–139 a. a. basic proteins (pI ~10). All Asr homologues possess at least one conservative 9 a. a. acid shock motif QKAQAAKKH located near the protein N-terminus. The motif was found repeated a different number of times (1–4) in Asr homologues of enterobacteria.

We have recently observed novel phenotypes associated with *E. coli asr* gene function. The *E. coli asr* gene has been found to be required for competitive colonization of the lower intestine of animal host. We have also demonstrated that *E. coli asr* is important for bacteria competing for survival in

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the stationary phase [2]. These findings indicate that the *asr* gene is involved in a broader spectrum of adaptive responses than acidity and is significant for enterobacterial survival in the primary and secondary habitats.

The *Asr* gene of *Enterobacter cloacae* represents the closest homologue of *E. coli asr* among enterobacteria. *E. cloacae* is part of the normal flora of gastrointestinal tract of many people and is widely distributed in various environmental niches. *E. cloacae* is also an opportunistic pathogen able to cause nosocomial infections with the mortality rate reported to be highest among all *Enterobacter* infections [11]. Therefore, the *E. cloacae asr* gene might play an important role in the adaptive responses and host colonization of this opportunistic pathogen.

To address this question, we have examined whether the *E. cloacae asr* gene is functional *in vitro* under acid stress conditions as well as *in vivo* during mouse gastrointestinal tract colonization.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains were MC4100 (F⁻*araD139* Δ(*argF-lac*)205 *flbB5301 ptsF25 relA1 rpsL150 deoC1*) [12], and MC4100*asr* [10]. The plasmids used in the study – pGB2 [13], pAS2 [10], pEC5 [10] and pLEC5 – had been constructed earlier [10]. Bacteria were grown in Luria–Bertani (LB) medium, unless otherwise indicated. If needed, antibiotics were added at the following concentrations: streptomycin sulphate 50 μg/ml, kanamycin 60 μg/ml, spectinomycin 50 μg/ml, ampicillin 100 μg/ml.

Acid stress. Cells from overnight cultures were diluted 1 : 1000 with a low phosphate medium (LPM) [14] buffered with MOPS (pH 7.0) to a final concentration of 0.1 M. Cells were grown at 37 °C with rotary aeration to an OD A_{590} of 0.5 and then resuspended in the same volume of LPM medium adjusted by HCl to pH 4.5. After incubation for 1 h at 37 °C with rotary aeration, the cells were collected by centrifugation and prepared for PAGE as described elsewhere [15].

Animal gastrointestinal colonization experiments. The relative fitness of *E. coli* strains for colonization of the mouse intestine was assayed as described in [16, 17] with minor changes. Briefly, five to seven week old BALB/c mice were given drinking water containing streptomycin sulphate (5 g / l) for 24 h prior to ingestion of bacteria in order to eliminate resident facultative bacteria. Following 3 h of starvation for food, each mouse was fed 50 μl of 10 % (wt / vol) sucrose in PBS containing 10^7 CFU of suspended exponential phase bacteria. For competition experiments, the strains were mixed 1 : 1 prior to ingestion, yielding 10^7 CFU of each strain in the same volume. After the bacterial suspension had been ingested, food was returned to the mice. Streptomycin containing water was given to the mice throughout the experiment. 0.5 g of feces, not older than 6 h, was homogenized, serially diluted and plated onto LB agar plates containing appropriate antibiotics. CFU was counted after incubation at 37 °C for 14–16 h. Experiments were repeated at least three times. The $\log_{10}$ mean number of CFU per gram of feces and the standard error of the $\log_{10}$ mean of the CFU per gram of faeces were calculated for each set of mice at each time point.

Protein electrophoresis and Western analysis. Proteins were fractionated using 20% SDS PAGE and stained with Coomasie briliant blue dye as described elsewhere [15, 18]. For Western analysis, *E. coli* proteins were separated by SDS PAGE, then fractionated proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond C Extra, Amersham). Western analysis was performed as described elsewhere [15], using polyclonal rabbit antibodies against *E. coli* Asr-His_6 diluted 1 : 500 [9]. Binding of the primary antibody was evaluated by incubating with goat horseradish peroxidase anti-rabbit immunoglobulin G conjugate (Amersham).

RESULTS

Enterobacter cloacae asr is the closest *Enterobacteriaceae* homologue of *E. coli asr*. The open reading frame deduced from the *E. cloacae asr* DNA sequence is capable to code for a protein of 102 amino acids (Fig. 1). The amino acid sequence of the *E. cloacae* Asr polypeptide displays the highest (76%) amino acid identity to *E. coli* Asr polypeptide as judged from

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                10          ▽       30        40        50
ECOLI   MKKVLALVVAAAMGLSSAAFAAETTTTPAPTATTTKAAPAKTTHHKKQHKAAP--- 53
ENTCL   MKKVLALVVAAAMGLSSAAFAAETTATAAPAAST--AAPAKTVHHKKHHKAAKPAA 54
        *************************:*.**:*:*  ******.****:****

            ▼60         70         80         90         100
ECOLI   AQKAQAAKKHHKNTKAEQKAPEQKAQAAKKHAKKHSHQQPAKPAAQPAA 102
ENTCL   EQKAQAAKKHHK--KAAKPAVEQKAQAAKKHHKKAAKHEAAKPAAQPAA 101
         **********  ** : * ********** ** :::::.*********
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Fig. 1. Sequence alignment of Asr proteins of *E. coli* and *E. cloacae*. ECOLI – Asr of *E. coli* (P36560), ENTCL – Asr of *E. cloacae* (Q93MH6). The cleavage site of the *E. coli* Asr signal peptide and the cleavage site of the 8 kDa polypeptide are indicated by open and full arrowheads, respectively. Numbers above the aligned sequences denote every tenth amino acid of Asr of *E. coli*. Identical amino acids are indicated by asterisks, conserved substitutions are indicated by colons, and semi-conserved substitutions are indicated by dots. Sequences were aligned using ClustalW2

a comparison of open reading frames deduced from sequences of *asr* gene homologues of *Enterobacteriaceae* (data not shown). The length of *E. coli* and *E. cloacae* Asr polypeptides differ by one amino acid only (Fig. 1). The translational start codon of *E. cloacae* Asr polypeptide overlaps with the experimentally determined translational start for *E. coli* Asr protein [10].

Both *E. coli* and *E. cloacae* Asr homologues possess an identical N-terminal signal peptide consisting of 21 amino acids (Fig. 1) and two acid shock motives QKAQAAKKH consisting of 9 amino acids (Fig. 1, underlined). We have shown previously that signal peptide is removed from *E. coli* Asr protein precursor in acid-induced *E. coli* cells (pH 4.5) (Fig. 1, indicated by open arrowhead). We have also observed the second *E. coli* Asr protein cleavage site within the first acid shock motif relative to N-terminus generated by an unknown protease (Fig. 1, indicated by full arrowhead) [10]. The latter cleavage is important for *E. coli* Asr protein function as the deletion of the amino acids at the cleavage position resulted in an acid-sensitive phenotype [10]. As the *E. cloacae* Asr polypeptide possesses identical acid shock protein motives present in *E. coli* Asr polypeptide, we next asked whether it undergoes proteolytical cleavage in acid-induced *E. coli* cells.

Enterobacter cloacae Asr protein undergoes proteolytic processing under acid-stress conditions

To examine the synthesis of *E. cloacae* Asr protein in acid-induced *E. coli* cells, we have introduced the high copy plasmid pEC5 harboring the *E. cloacae asr* gene with its own promoter and terminator sequences [10] into the *E. coli asr* mutant strain MC4100*asr* (see Materials and Methods). MC4100*asr* cells harboring pEC5 plasmid or pAS2 plasmid with the cloned *E. coli asr* gene were grown in LPM medium, pH 7.0, until the mid-logarithmic phase as described in Materials and Methods. Cells were acid-shocked (pH 4.5) for 1 h, and proteins were analysed by SDS PAGE and Western analysis, using antibodies against *E. coli* Asr-His_6 recombinant protein (see Materials and Methods).

As can be seen in Fig. 2 (lanes 1 and 3), when exponentially grown endogenous *asr* gene-deficient *E. coli* cells harboring either pAS2 or pEC5 plasmid are shifted to pH 4.5 for 1 h, *E. coli* and *E. cloacae* Asr polypeptides are synthesized as ~18 kDa preproteins. In consistency with our earlier observations, *E. coli* Asr undergoes cleavage of the signal peptide and a second cleavage within the first conservative acid shock protein motif yielding polypeptides with apparent molecular masses of ~15 kDa and 8 kDa, respectively (Fig. 2, lane 1).

The *E. cloacae* Asr polypeptide displays an identical cleavage profile in acid-induced *E. coli* cells, except that ~15 kDa polypeptide corresponding to the polypeptide with the removed signal sequence is not observed (Fig. 2, lane 3). Most likely, *E. cloacae* protein after its transport into periplasm undergoes rapid cleavage at the second cleavage site, yielding 8 kDa polypeptide (Fig. 2, lane 3). A similar rapid processing of Asr precursor was observed in *E. coli*, which contained a chromosomal *asr* gene copy only, where intermediate preproteins of 18 kDa and 15 kDa were not observed in acid-induced bacterial cells [10].

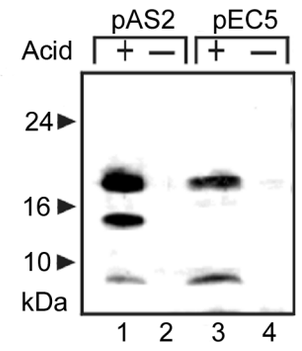

Fig. 2. Expression of Asr homologous genes under acid stress. *E. coli* MC4100*asr* strains with pAS2 or pEC5 plasmids were grown in LPM (pH 7.0) to mid-exponential phase and then shifted to pH 4.5 for 1 h as described in Materials and Methods. Whole cell lysates (30 µg protein) were separated by 20% SDS PAGE, and Western analysis was performed using anti-Asr(His_6) antibodies as described in Materials and Methods. The positions of protein molecular mass standards (kDa) are indicated by arrowheads on the left

A similar processing profile leading to an accumulation of polypeptide with the apparent molecular mass of 8 kDa in *E. coli* cells harboring either *E. cloacae* or *E. coli asr* homologues and the high similarity of their amino acid sequences suggest that the second cleavage most likely occurs between Q59 and A60 residues within the acid shock motif of *E. cloacae* Asr (Fig. 1, indicated by full arrowhead).

Observations that *E. cloacae* Asr protein displays a similar processing profile *in vitro* under acid shock conditions prompted us to test whether it might functionally replace *E. coli* Asr protein *in vivo*.

E. cloacae asr gene complements *E. coli asr* deficiency during competitive colonization of the mouse lower intestine

According to our earlier observations, the *E. coli asr* gene is required for bacterial growth at a moderate acidity (pH 4.5) as well as for the induction of acid tolerance at a moderate acidity. Inactivation of *asr* results in an acid-sensitive *E. coli* phenotype [10]. Moreover, our recent investigations have revealed novel phenotypes associated with the *E. coli asr* gene when bacteria invade the gastrointestinal tract which is their primary habitat. The *E. coli asr* deficient strain was unable to colonize mouse lower intestine when grown in competition with its parent. Introduction of a low copy plasmid with an *E. coli asr* gene copy into the MC4100*asr* strain largely restored the colonization-deficient phenotype [2], clearly indicating that *asr* function is required to colonize the mouse lower intestine under competition.

Based on these observations, we asked whether the *asr* gene of *E. cloacae* is able to complement the colonization-deficient phenotype of the *E. coli asr* mutant. We assumed that

asr function could be important for enterobacterial pathogens to invade the host.

We examined the ability of the endogenous *asr* gene-deficient *E. coli* strain harboring the low copy plasmid pLEC5 with the cloned *E. cloacae asr* gene [10] to colonize mouse gastrointestinal tract. A streptomycin-treated animal model was chosen as it is useful for studying the ability of the strains to colonize the lower intestine [16, 19]. In streptomycin-treated mice, most of facultative aerobes (mostly gram-negative bacteria) and some anaerobe populations are removed, therefore a niche for the introduced strain is created without any observable damage to the animal. The animals used for experiments were BALB/c inbred mice 5–7 weeks old. The amount of bacteria fed to the mice was determined empirically, the best concentration being 10^7 bacterial cells per mouse (results not shown).

E. coli MC4100*asr* strain harboring pLEC5 plasmid with the functional *E. cloacae asr* gene and MC4100 strain were fed together in equal numbers to streptomycin-treated mice (10^7 CFU of each strain per mouse) (see Materials and Methods). In another experimental set, 10^7 CFU of *E. coli* MC4100*asr* cells harboring the control vector pGB2 were fed to mice together with an equal amount of MC4100 cells. We had shown previously that when fed separately to mice, *E. coli asr* mutant strain and its parent showed equal colonization profiles [2].

As one can see in Fig. 3 A, B, at the first time point of testing, both strains could be detected in the feces (approximately 6 h after feeding) at approximately 10^6 CFU/g. The presence of *asr* mutation in *E. coli* MC4100 did not influence the ability of the strain to pass the stomach and start colonization (Fig. 3 A, B). However, for the following course of experiment, the MC4100*asr* strain harbouring the control vector pGB2 was rapidly decreasing in number, reaching a 10^3-fold difference after 6 days. The *asr* mutant strain was found fully eliminated from the mouse intestine by day 10.

Conversely, both the wild type *E. coli* strain and the MC4100*asr* strain harbouring the pLEC5 plasmid with the *E. cloacae asr* gene were found in equal counts for the major part of experiment until day 10 (Fig. 3 B). In the last days of the experiment, a decrease of MC4100 strain was noticed, resulting in a 100-fold decline of the numbers of the wild type strain. Thus, a complementation with an *E. cloacae asr* homologue copy resulted in an entirely restored wild type phenotype or, furthermore, a more competitive phenotype than MC4100.

All plasmid-bearing strains were checked for the maintenance of plasmids throughout and in the end of experiments, and no plasmid loss was observed (results not shown).

DISCUSSION

Adaptive bacterial responses include a wide range of molecular mechanisms ranging from conservative through genera to highly species-specific [20]. Members of the family *Enterobacteriaceae* are a normal part of the gut microflora found in the intestines of humans and other animals, while others are present in water or soil or are parasites on a variety of different animals and plants. The family is most famous for many of the more familiar pathogens that cause gastroenterological, urological and respiratory diseases. The enterobacteria possess an amazing ability to survive at fluctuating pH inside the gastrointestinal tract of the host as well as in the environment outside the host [21, 22]. As a result, bacteria have

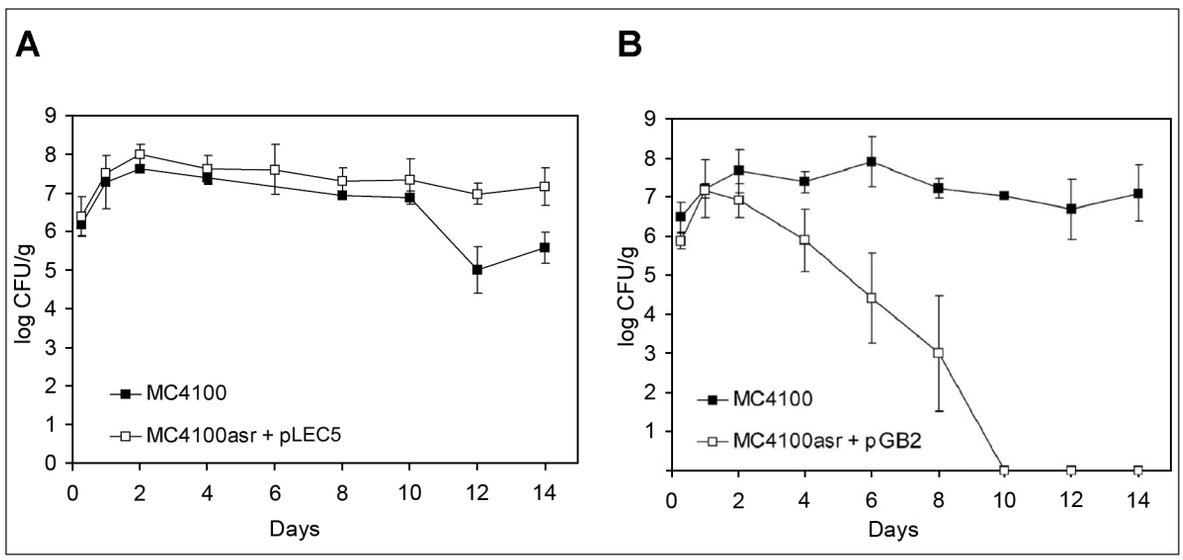

Fig. 3. Complementation of *asr* phenotype in mouse gastrointestinal tract using low copy plasmids with *E. cloacae asr* gene. 10^7 CFU of indicated *E. coli* strains were fed to streptomycin-treated mice, mixed in equal amounts. At indicated times, fecal samples were collected, homogenized, diluted and plated as described in Materials and Methods. Data points are the mean values of at least three experiments, bars indicate standard error. (A) MC4100 with MC4100*asr* pLEC5, (B) MC4100 with MC4100*asr* pGB2

evolved complex strategies designed to adapt to acid stress [20]. The presence of such strategies has been shown to correlate with the infectious dose of the pathogens [5, 23–25].

The acid-inducible *asr* gene appears to be a conservative element of *Enterobacteriaceae* adaptive response. First, its transcription is strongly triggered by a low environmental pH in enterobacterial species both *in vitro* [1, 10] and *in vivo* [26, 27]. Second, promoters of *asr* gene homologues contain a conservative regulatory region which has been demonstrated to be important for acid induction [1, 28]. Third, as shown in this study, *Enterobacteriaceae asr* gene homologues could replace each other *in vivo*, restoring the loss of gene function during a competitive colonization of animal gastrointestinal tract. Interestingly, the latter observation is not obviously related to gene involvement in acid-stress response since the *E. coli asr* mutant and its parent were shown to pass the acidic environment of the mouse stomach in equal numbers 6 h after feeding (Fig. 3 A). While dispensable when passing the acid barrier in the mouse stomach, the *E. coli asr* gene was required for competition in the intestine, as the *asr* mutant harbouring the control vector pGB2 was unable to start colonization when grown with its parent (Fig. 3 B). In the streptomycin-treated mouse model, the initial stage of bacterial growth in the intestine reflects the ability of competing strains to use the preferred nutrients [29, 30]. Chang et al. [29] used the whole-genome expression profiling of *E. coli* grown on mucus, which mimicked the nutrient environment in the mammalian intestine, and did not observe significantly altered expression of *asr* as compared with cells grown in minimal glucose medium. Most of the induced genes where those coding the components of catabolic pathways for nutrients present in the mucus. However, recent observations indicate that numerous other aspects of colonization process, such as respiratory flexibility, motility, adhesion, resistance to stress, appear to be important [31–33]. It cannot be ruled out that wild type bacteria induce the *asr* gene when passing the acidic stomach, leading to an advantage in the initial colonization of the mouse intestine. The presence of the *E. coli asr*-specific transcript was observed by RT-PCR in bacteria collected from mice fecal pellets 48 h after feeding [2].

Complementation experiments performed with an *E. coli asr*-deficient strain harbouring a low copy plasmid with the *E. cloacae asr* homologue show the importance of the functional *asr* for maintaining enterobacteria in the intestine of the host in a highly competitive situation. The ability of pathogenic enterobacteria to persist in the intestine could be facilitated by similar processes.

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ENTEROBACTER CLOACAE RŪGŠTINIO STRESO *ASR* GENO REIKŠMĖ PELIŲ ŽARNYNO KOLONIZACIJAI

Santrauka

Escherichia coli asr genas ir jo ortologai *Enterobacteriaceae* šeimos bakterijų genomuose yra adaptacinio atsako elementai, kurių raišką labai veiksmingai aktyvinantis signalas yra žemas aplinkos pH (pH < 5.0) [1]. Genai koduoja nedidelius bazinius 82–139 aminorūgščių liekanų baltymus, kurių funkcija nežinoma. *E. coli asr* genas yra reikalingas bakterijų augimui esant žemam augimo terpės pH (pH 4.5) ir svarbus bakterijai įsitvirtinti žinduolių žarnyne konkurencijos sąlygomis [2]. Šiame darbe, naudodami konkurencinį pelių modelį, atskleidėme, kad bakterijos *Enterobacter cloacae asr* genas gali funkciškai pakeisti *E. Coli asr* geną, grąžindamas mutantinio kamieno, turinčio išveiklintą geną, prarastą gebėjimą konkurencijos sąlygomis kolonizuoti pelių žarnyną. *E. cloacae* ir *E. coli asr* genų koduojami baltymai turi 76% vienodų aminorūgščių liekanų ir yra labiausiai tarpusavyje panašūs tarp enterobakterijų Asr baltymų. Panašus *E. cloacae* ir *E. coli* Asr baltymų brendimas rūgštinio streso sąlygomis ir *E. cloacae asr* geno gebėjimas funkciškai pakeisti *E. coli* geną liudija, kad enterobakterijų *asr* genas yra konservatyvus adaptacinio atsako dalyvis, svarbus joms įsitvirtinant gyvūnų žarnyne.