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Identification and characterization of the new gene *rhtA* involved in threonine and homoserine efflux in *Escherichia coli*

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Abstract

The *rhtA* gene known as the *ybiF* ORF in the genome of *Escherichia coli* was identified as a new gene involved in threonine and homoserine efflux. This gene encodes a highly hydrophobic membrane protein that contains 10 predicted transmembrane segments. The *rhtA23* mutation, which is an A-for-G substitution at position −1 in relation to the ATG start codon, increases the expression level of the *rhtA* gene. The overexpression of *rhtA* gene results in resistance to inhibitory concentrations of homoserine, threonine and a variety of other amino acids and amino acid analogues, reduced threonine and homoserine accumulation in resistant cells and increased production of threonine, homoserine, lysine and proline by the respective producing strains. The RhtA protein belongs to a vast family of transporters. The genome of *E. coli* contains at least 10 paralogues of RhtA. Phylogenetic analysis indicates that a common ancestor of living organisms contained several RhtA homologues.

Keywords: Efflux; Transport; Membrane protein; Homoserine; Threonine

1. Introduction

The growth of a number of microorganisms is inhibited in minimal media containing some natural amino acids; such inhibition is usually reversed by other amino acids. The nature of this phenomenon has only been studied for a few cases. Exogenous amino acids can suppress common steps of a biosynthetic pathway and cause starvation for other amino acids. For example, in *Escherichia coli* K-12, growth inhibition by valine is caused by the suppression of isoleucine biosynthesis. Exogenous valine inhibits the activity of the sensitive acetolactate synthases (EC 4.1.3.18) that participate in the synthesis of both valine and isoleucine [45]. Growth inhibition caused by serine and cysteine is associated with the suppression of theonine and isoleucine biosynthesis. Serine inhibits homoserine dehydrogenase I (EC 1.1.1.3), which is involved in the biosynthesis of theonine and isoleucine [13]. Cysteine inhibits threonine deaminase (EC 4.21.16), which is involved in the conversion of threonine to α-ketobutyrate, the first reaction of the isoleucine biosynthesis pathway [14]. Another example is growth suppression by homoserine, the metabolic precursor of threonine and methionine. Homoserine seems to inhibit the activity of NADP-dependent glutamate dehydrogenase (EC 1.4.1.4), an enzyme that catalyzes the initial reaction in inorganic ammonium assimilation [18].

Selection of the mutants resistant to the inhibitory action of amino acid analogues and amino acids was for a long time a routine approach in the breeding of bacterial strains overproducing amino acids. The resistance to the inhibitors was mainly attributed to a release of the respective biosynthesis pathway from the feed-back regulation [15,46]. However, during the past two decades, numerous reports have accumulated on a mechanism of resistance to toxic compounds that involves membrane proteins capable of extruding inhibitors from the cytoplasm [27,35]. Krämer and co-workers obtained biochemical and physio-

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logical data on the presence in bacteria of carrier proteins responsible for the efflux of amino acids [19]. One such carrier, LysE, a lysine–arginine exporter of *Corynebacterium glutamicum*, has recently been identified and functionally characterized [5,48]. We identified the *rhtB* and *rhtC* genes of *E. coli* that encode proteins involved in homoserine and threonine efflux [51]. These proteins together with LysE belong to a large superfamily of transporters [2]. More recently, a novel efflux pump of *C. glutamicum*, ThrE, specific for threonine and serine, has been described [38]. In addition, Daßler et al. [6] identified the YdeD protein, which is involved in efflux of metabolites of the cysteine pathway. The YdeD and ThrE proteins belong to new families of transporters [6,50].

In the course of our overall work to improve a threonine-producing strain of *E. coli*, we found that this strain was sensitive to the presence of threonine when grown in minimal medium. We isolated a mutant of this strain, T23, resistant to the inhibiting concentrations of threonine, which proved to be an excellent threonine producer with a markedly increased yield [7]. This mutant, now renamed RhtA23, also appeared to be resistant to homoserine. In the present work, we report on the identification and characterization of the *rhtA* gene, previously known as *ybiF* ORF in the *E. coli* K-12 genome. This gene, when overexpressed, confers upon cells resistance to high concentrations of threonine, homoserine and a variety of other amino acids and amino acid analogues, and promotes threonine and homoserine excretion.

2. Materials and methods

2.1. Bacterial strains, plasmids, phages, growth and amino acid fermentation conditions

Bacterial strains, plasmids and phages used in this study are listed in Table 1. *E. coli* cells were grown at 30 °C or 37 °C in LB or M9 minimal medium [26] supplemented with 40 μM thiamine HCl, 0.4% (wt/vol) D-glucose and, when required, with amino acids (40 μg ml^{-1}) and antibiotics at the following concentrations: kanamycin, 50 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; streptomycin, 200 μg ml^{-1}; and ampicillin, 100 μg ml^{-1}. Solid media were prepared by adding a 1.2% (wt/vol) agar to the liquid broth. For uptake studies bacteria were grown in medium A [26] supplemented with 40 μM thiamine HCl and 0.5% (wt/vol) sodium succinate. Amino acids and amino acid analogues were obtained from Sigma, except for glutamate (monosodium salt) obtained from Ajinomoto (Japan), and L-homoserine produced at the State Research Institute of Genetics and Selection of Industrial Microorganisms and purified (>99.5%) at the Laboratory of Analytical Chemistry of the aforementioned Institute. L-[G-^{3}H]homoserine and L-[G-^{3}H]threonine were prepared in the Institute of Molecular Genetics (Moscow).

2.2. Selection of the threonine-resistant mutants and study of phenotypes

The mutants resistant to threonine were selected in the M9 medium with threonine (40–50 mg/ml) after the treatment with N-methyl-N′-nitro-N-nitrosoguanidine [26]. The minimal inhibitory concentrations (MICs) of amino acids and amino acid analogues for each strain were determined on M9 minimal agar plates containing graded concentrations of an inhibitor. The agar plates with very high amino acid concentrations were prepared by adding a dry amino acid substance to the hot agar medium. The plates were spotted with 10^4 to 10^5 cells from an overnight culture grown in a minimal medium (supplemented with ampicillin for plasmid strains). The MIC was determined as the lowest concentration of inhibitor preventing the growth after 44 h incubation at 37 °C. The experiments were repeated three to five times and revealed consistent MICs. The MICs of antibiotics were determined using the LB agar plates as above.

2.3. Determination of threonine and homoserine accumulation in cells

The amino acid accumulation in cells was examined as described previously [49]. Cells from mid-exponential-phase cultures were harvested by centrifugation, washed three times and suspended in 50 mM potassium phosphate buffer (pH 6.9) containing 0.5 mM $MgSO_4$ (buffer A). The assay mixture contained cell suspension in buffer A (0.3 mg protein per ml), 0.5% (wt/vol) D-glucose, 100 μg of chloramphenicol per ml, and 40 mM NaCl. Reaction mixtures were preincubated for 15 min at 37 °C prior the addition of substrates. ^{3}H-labeled substrates were added to the following specific activities and final concentrations: L-[G-^{3}H]homoserine (90 mCi/mmol) 16.5 μM; L-[G-^{3}H]threonine (150 mCi/mmol) 10 μM. Samples (100 μl) were removed at intervals, and the cells were collected by vacuum filtration on membrane filters (0.45-μm pore size, Schleicher–Schuell) that had been presoaked in buffer A containing 300 μg of chloramphenicol per ml. The filters were immediately washed with 5 ml of ice-cold buffer A, and the radioactivity on the filters was counted in a scintillation counter. The results were calculated in relation to the protein content. Protein concentration was determined using Bicinchoninic acid kit for protein determination (Sigma) and bovine serum albumin as a standard. All experiments were performed at least in triplicate, and the results of typical experiments are displayed in the figures.

2.4. Measurement of threonine intracellular pools

The intracellular pools of threonine were determined essentially as described previously [8] with some modifications. The cells were harvested in the exponential growth

Table 1
E. coli strains, plasmids and phages

	Genotype/description	Reference/source
Strains		
TG1	*supE hsd*Δ5*thi*Δ(*lac* – *proAB*) *F′*(*traD36 proAB*$^+$ *lacI*q *lacZ* ΔM15)	[35]
BL21(DE3)	F-*ompT[lon] hsdS*$_B$ (r-m-; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
MG442	F$^-$ *thrA442 ilvA442*	[12]
W3350	F-*galKT* IN(*rrnD-rrnE*)	VKPM[a]
N99	W3350 *rpsL*	VKPM[a]
N99 *rhtA23*	W3350 *rpsL rhtA23*	This work
B-6105	*thr*::Tn10	VKPM[a]
N99 *rhtA23 thr*::Tn10	W3350 *rpsL rhtA23 thr*::Tn10	This work
C600	*thi1 thrB1 leu6 lacY1 supE44 tonA21 rpsL rfbD1*	[36]
NZ10	C600 *leu*$^+$	[51]
Gif 102	*thrA*$_2$*1015 metLM1005 lysC1004*	[42]
Gif 102 *rhtA23*	*thrA*$_2$*1015 metLM1005 lysC1004 rhtA23*	This work
VL2151	W3350 Δ*putAP*, Tn10 DH-Pro^R	[22]
VL2151 *rhtA23*	W3350 Δ*putAP*, Tn10 DH-Pro^R rhtA23	This work
VL614	*thrA*$_2$*1015 metLM1005 lysC1004 AEC*^{R}	[22]
VL614 *rhtA23*	*thrA*$_2$*1015 metLM1005 lysC1004 AEC*^{R} *rhtA23*	This work
Plasmids		
pUC21	Ap^R	[47]
pBlueskript-KS$^+$	Ap^R	Stratagene
pUC4K	Ap^R Km^R	Promega
pJEL246	Ap^R *lacZ′YA*	P. Valentin–Hansen (via A.S. Mironov)
pAL4	Ap^R *thrA*	VKPM[a]
pAZ1	Ap^R translational fusion *rhtA′–′lacZ′YA*	This work
pAZ2	Ap^R translational fusion *rhtA23′–′lacZ′YA*	This work
pVIC40	Sm^R, *thrA*$_{442}$*BC*	[7]
pNPZ10R	Mu d5005 with 16.5-kb insert of MG442 *rhtA23* chromosome DNA	This work
pNPZ4S	Mu d5005 with 9.3 kb insert of MG442 chromosome DNA	This work
pNPZ14	*Sac*II-*Hin*dIII fragment of pNPZ4S cloned into pBlueskript-KS$^+$	This work
pNPZ16	*Xmn*I-*Ava*III fragment of pNPZ4S (*rhtA*$^+$) into pBlueskript-KS$^+$	This work
pNPZ17	*Xmn*I-*Ava*III fragment of pNPZ10R (*rhtA23*) into pUC21	This work
pNPZ18	*Sna*BI-*Hin*dIII fragment of pNPZ16 into pUC21	This work
pNPZ30	*Hin*cII-*Hin*cII fragment (*kan*) of pUC4K in *Eco*47III of pNPZ16	This work
pET22b	Ap^R	Novagen
pET22b-rhtA	Ap^R, *rhtA*	This work
Phages		
P1 *vir*		VKPM[a]
Mu d5005	Km^R	[11]
Mu *c*ts	Mu *c*ts*62*	[11]

[a] Russian National Collection of Industrial Microorganisms. Ap^R, ampicillin resistance; Sm^R, streptomycin resistance; Km^R, kanamycin resistance; DH-Pro^R, 3,4-dehydro-DL-proline resistance; AEC^R, S(2-aminoethyl)-L-cysteine resistance.

phase ($OD_{600} = 0.6$), separated from the medium by silicone oil centrifugation and extracted by 20% perchloric acid. The extracts were homogenized by sonication and then neutralized with 2 M Na_2CO_3 and centrifuged through microcentrifuge filters (0.5-μm pore size, type HYC PTFE, Nihon Millipore Ltd., Yonezawa, Japan). The samples of filtrates after appropriate dilution with HCl (final HCl concentration was 0.02N) were applied to an amino acid analyzer (Biotronic LC 2000, Puchheim, FRG) for determination of threonine concentration. The results were calculated in relation to cell dry weights (DW). Mean data from three independent experiments are presented.

2.5. Determination of amino acid excretion in a short-term fermentation

Cells of the respective producing strains were harvested in the exponential growth phase, washed three times with ice-cold M9. Efflux was initiated by suspending the cells in the prewarmed M9 medium supplemented with 40 μM thiamine HCl and 0.4% (wt/vol) D-glucose at a cell density of 1–2 mg protein per ml. Samples (100 μl) were removed at regular intervals; the cells were separated by centrifugation through microcentrifuge filters. The samples of filtrates after appropriate dilution were applied to the amino acid

analyzer as above. All experiments were performed at least in triplicate, and the results of typical experiments are displayed in the figures.

2.6. Determination of amino acid production

For threonine and homoserine production in pH-controlled cultivation using laboratory fermenters (Marubishi Co. Ltd., Japan) the medium and conditions described previously were used [7], except for glucose added as a carbon source instead of sucrose. L-threonine (0.5 g/l) was added to the medium, when homoserine-producing strains were used. For proline and lysine production tube fermentations were performed. In these experiments we used the following fermentation medium: 50 g glucose, 10 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 0.6 g NaCl, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4 \cdot 5H_2O$, 0.0002 g thiamine-HCl, 2 g yeast extract, and 20 g $CaCO_3$ per liter. L-homoserine (0.3 g/l) was added to the medium, when lysine-producing strains were used. The strains were cultivated in glass tubes 20 × 200 mm at 37 °C for 46 h with a rotary shaker. All experiments on amino acid production were repeated at least three times, and mean data are presented.

2.7. Genetic methods and DNA manipulation

Cloning of the *rhtA* gene was conducted using phagemid Mu d5005, according to the technique described by Groisman and Casadaban [11]. As donors of bacterial chromosome, strains MG442 and MG442 *rhtA23* lysogenic for Mu *c*ts were used. The phagemids that had insertions were selected using M9 minimal medium that contained kanamycin and homoserine (10 mg/ml). Conjugation and transduction by the P1*vir* phage were conducted according to Miller [26]. For the selection of *rhtA23* transductants minimal medium containing 10 mg/ml homoserine was used. No spontaneous mutants resistant to homoserine arise under this condition.

All manipulations with recombinant DNA were carried out according to standard procedures [36] and the recommendations of enzyme manufacturers (MBI Fermentas or New England Biolabs). For sequencing of the *rhtA* and *rhtA23* alleles, restriction fragments of plasmids pNPZ16 and pNPZ17 were subcloned into the pUC21 vector. Sequencing was performed by Sanger's dideoxynucleotide method on both strands using universal vector primers, and by automatic sequencing with an Abi Prism 377TM DNA sequencer (Applied Biosystems).

2.8. Construction of the pAZ1 and pAZ2 plasmids and β-galactosidase activity assay

Plasmids pAZ1 and pAZ2 containing a translational fusion of the 5′-terminal fragments of wild type (*rhtA*$^+$) and mutant (*rhtA23*) alleles of the *rhtA* gene, respectively, with the *lacZ*′ gene deficient of the promoter, SD sequence and 9 initial triplets of the coding region were constructed as follows. Plasmid pJEL246 harboring *lacZ*′*YA* genes was used as a vector. The 5′-terminal fragments of *rhtA*$^+$ and *rhtA23* containing 289 nucleotides upstream of the start codon and 8 initial triplets of the *rhtA* gene were isolated from pNZ16 and pNZ17, respectively, using *Xmn*I and *Cfr*10I restriction sites. Next, after intermediate cloning, fragments were cut out using *Eco*RI–*Bgl*II sites and inserted into *Eco*RI–*Bam*HI sites of the pJEL246 vector giving in frame fusion with *lacZ*′ (Fig. 2A). The structures of the fusion regions were confirmed by sequencing. For a β-galactosidase activity assay, strains were grown in LB medium at 37 °C. Measurements and conversion of the activity values with regard to the protein unit were conducted as described by Miller [26]. The β-galactosidase activity assay was repeated three times with independent cultures, and each experiment gave similar results. Representative data are shown.

2.9. Insertional inactivation of the rhtA gene

The 1.3 kb *Hin*cII fragment containing the kanamycin-resistance gene (*kan*) was isolated from pUC4K and inserted into the *Eco*47III site of pNPZ16, giving pNPZ30. The integration of the obtained insertion mutation into the chromosome of strain N99 was conducted according to Parker and Marinus [31]. The substitution of the wild type allele of the *rhtA* gene for the *rhtA*::*kan* insertion mutation was confirmed by genetic mapping and Southern hybridization [36].

2.10. Construction of the pET22b-rhtA plasmid and determination of cellular localization of the rhtA gene product

The *rhtAA* coding sequence was amplified from pNPZ16 by the polymerase chain reaction (PCR) using primers: 5′-agaa**catatg**cctggttcattacgtaaaa-3′ (*Nde*I site introduced for cloning is in bold) and 5′-gtgc**gaattc**taaatttaattaatgtctaa-3′ (*Eco*RI site introduced is in bold). The PCR product was digested with *Nde*I and *Eco*RI and inserted into the bacterial expression vector pET22b cut with *Nde*I and *Eco*RI. The resulting plasmid pET22b-rhtA was transformed into *E. coli* strain BL21(DE3), which contains the T7 RNA polymerase gene under the control of the *lac* promoter. The induction of the T7 RNA polymerase gene and labelling of the protein with [^{35}S]methionine were carried out essentially as described previously [40]. The modifications were that protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration 2 mM) to 5 ml of a log-phase culture in M9 medium supplemented with 18 amino acid mixture (final concentration 0.005%). Labelling of the newly synthesized protein was performed by adding 50 μCi [^{35}S]methionine. Cells were harvested by

centrifugation and used for fractionation. The pellet was resuspended in a breaking buffer (100 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA, 2 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and cells were disrupted by sonication. After removal of the cell debris by centrifugation at 15 000 *g* for 20 min, membranes were collected by centrifugation at 180 000 *g* for 180 min. The resulting pellet (membrane fraction) was stirred in the breaking buffer containing 1 M KCl for 30 min at room temperature and centrifuged at 180 000 *g* for 180 min. The supernatant (soluble factions) was precipitated by incubation with trichloroacetic acid (10% final concentration) at 4 °C for 30 min, centrifuged and washed with acetone. All pellets were resuspended in the same volume as the corresponding supernatants. Proteins were examined by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [21].

2.11. Data bank search and protein sequence analysis

The hydropathic characters of proteins were assessed by the method of Kyte and Doolitle [20]; the profile of transmembrane segments was determined using the HMMTOP program [44]. Data bank search was conducted using PSI-BLAST at the NCBI and using Gapped BLAST in unfinished microbial genomes [3]. Preliminary sequence data were obtained from the Institute for Genomic Research (http://www.tigr.org). Multiple sequence alignment was conducted using the CLUSTAL W programs [43]. For phylogenetic analysis unambiguously aligned 272 RhtA residues were used. The alignment is available on request via e-mail. The most parsimonious trees were obtained using PAUP 4.0.0d55, Wisconsin Package Version 8.1, Genetics Computer Group, Madison, WI, with the option of heuristic search (random sequence addition, Nreps = 10, Swap = TBR). The distance neighbor-joining trees were deduced using the PHYLIP package [9]. Maximum likelihood trees were inferred with PUZZLE-TREE 5.0 [39] using the mtREV24 model of substitution [1] and rate heterogeneity with 8 categories. The TreeView program, version 1.5 [30] was used for graphical presentation of the tree.

3. Results

3.1. Selection and genetic mapping of the rhtA23 mutation

We obtained a set of mutants from several *E. coli* K-12 strains capable of growth in minimal media with threonine at an inhibiting concentration. The majority of the mutants were found to be unstable and lost their resistance to threonine under non-selective conditions. One mutant was stable and also appeared to be resistant to homoserine. The mutation carried by this strain was designated *rhtA23* (resistance to homoserine and threonine). We found that this mutation can be transferred by phage P1-mediated transduction into different *E. coli* K-12 strains. Several *rhtA*$^+$ and *rhtA23* otherwise isogenic pairs of strains were constructed and used for further study. The *rhtA23* mutation was mapped within the 18.3 min region of the *E. coli* chromosome, close to the *glnHPQ* operon that encodes components of the glutamine transport system [28]. The frequency of *rhtA23* mutation co-transduction with the *glnP* and *glnH* genes was found to be 93% and 98%, respectively.

3.2. Cloning and identification of the rhtA gene

We cloned in vivo the wild type and mutant alleles of the *rhtA* gene, using the mini-Mu d5005 phagemid as described in Materials and methods. Recombinant phagemids conferring upon cells a resistance to high concentrations of threonine and homoserine were selected from the MG442 Mu *c*ts strain containing the *rhtA23* mutation, and from that containing the wild type (*rhtA*$^+$) gene. Restriction analysis showed that the phagemids obtained from both strains contained inserts belonging to different regions of the *E. coli* chromosome. One type of insert proved to be chromosome fragments from the region of 86 min. In this case, the resistance phenotype was related to overexpression of the *rhtB* and *rhtC* genes [51]. The second type of inserts contained chromosome fragments from the *glnHPQ* operon region. The respective phagemids were obtained both from the *rhtA23* mutant (pNPZ10R, Fig. 1), and from the *rhtA*$^+$ strain (pNPZ4S, Fig. 1). Multicopy vector subcloning of these inserts revealed that the minimal fragment conferring resistance had a length of 1.2 kb (plasmids pNPZ16 and pNPZ17, Fig. 1) and included the *ybiF* ORF (GenBank accession number AE000183) (Fig. 1). The ORF is preceded by an SD sequence (positions 8469-8473 in AE000183). The deletion of a fragment containing the 5′-end region of *ybiF* and 18 nucleotides downstream of the start codon (plasmid pNPZ18, Fig. 1) resulted in loss of resistance to threonine and homoserine.

Thus, we identified the *rhtA* gene as the *ybiF* ORF between the *pexB* and *ompX* genes and demonstrated that amplification of the wild-type allele of this gene also confers resistance to threonine and homoserine.

3.3. RhtA is an integral membrane protein

The *rhtA* gene encodes a protein that consists of 295 amino acid residues and has calculated molecular mass of 31.2 kDa. Analysis of the RhtA sequence revealed that it is a highly hydrophobic protein containing 10 predicted transmembrane segments (Fig. 2A). The N- and C-termini of the molecule are enriched with positively charged residues. Using the HMMTOP program we found that their putative location is related to the cytoplasm of cells. The hydropathy profile and the number of predicted transmembrane segments of RhtA are like those of the YdeD protein (Fig. 2A), which is involved in efflux of cysteine or cysteine-related compounds [6].

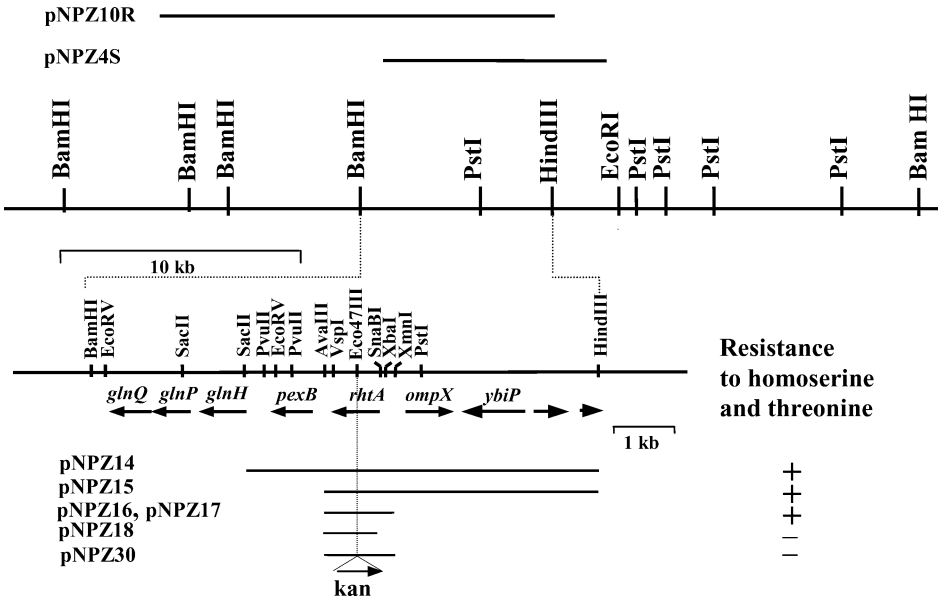

Fig. 1. Genetic and restriction map of the *E. coli* chromosome at 18 min and the plasmids harboring various DNA fragments of the region that confer resistance to homoserine and threonine.

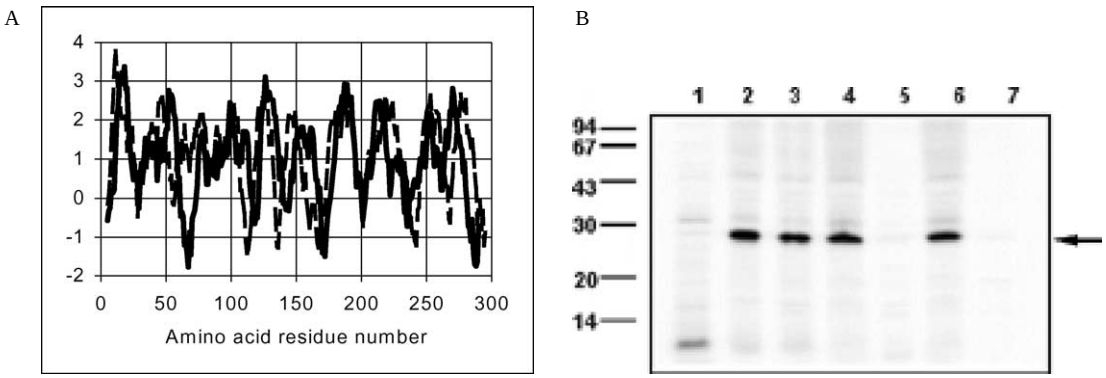

Fig. 2. (A) Hydropathy plot of RhtA (solid line) and YdeD (dashed line). The algorithm of Kyte and Doolittle [20] was used with a window span of nine. (B) Cellular localization of the RhtA protein. Each fraction of the differential centrifugation (see Materials and methods) was applied to 15% SDS polyacrylamide gel. Proteins were visualized by staining with Coomassie blue, and radioactivity was detected by autoradiography using a phosphorimager system (molecular dynamics). Lane 1, sediment of centrifugation at 15 000 *g* of the induced BL21(DE3) cells harboring the pET22b plasmid; lanes 2–7, equivalent volumes of the sediment and soluble fractions of RhtA expressing BL21(DE3) cells harboring pET22b-rhtA plasmid: 2, sediment of centrifugation at 15 000 *g*; 3, supernatant of centrifugation at 15 000 *g*; 4 and 5, sediment and supernatant, respectively, of centrifugation at 180 000 *g*; 6 and 7, sediment and supernatant, respectively, of centrifugation at 180 000 *g* after treatment of the membrane fraction with 1 M KCl. The molecular mass markers (in kDa) are indicated in the left margin. The arrow indicates RhtA.

To determine the cellular localization of RhtA, a specific labeling of the protein was carried out using the T7 RNA polymerase/promoter system followed by fractionation of the induced cells. The distribution of RhtA in the cellular fractions is shown in Fig. 2B. RhtA was co-sedimented with the membrane fractions (Fig. 2B, lane 4) and was absent in the fraction of soluble proteins (Fig. 2B, lane 5). The same protein distribution was observed after KCl treatment of the membrane fraction (Fig. 2B, lanes 6 and 7). These data support the notion that RhtA is an integral membrane protein. The electrophoretic mobility of RhtA corresponds to a molecular mass of approximately 25 kDa instead of the predicted 31.2 kDa, which may be a result of RhtA's hydrophobicity.

3.4. The rhtA23 *mutation is located beyond the coding region and affects the level of RhtA expression*

The wild type and mutant alleles of the rhtA gene were sequenced. The sequence of the wild-type allele was identical to that available from the GenBank database (GenBank accession number AE000183), and the mutant allele had an A-for-G substitution at the position −1 in relation to the ATG start codon of rhtA (Fig. 3A). The effect of the rhtA23 mutation on rhtA gene expression was determined using the translational fusions rhtA′–′lacZ and rhtA23′–′lacZ contained in the pAZ1 and pAZ2 plasmids, respectively (see Materials and methods). The TG1 strain was transformed by these plasmids, and β-galactosidase

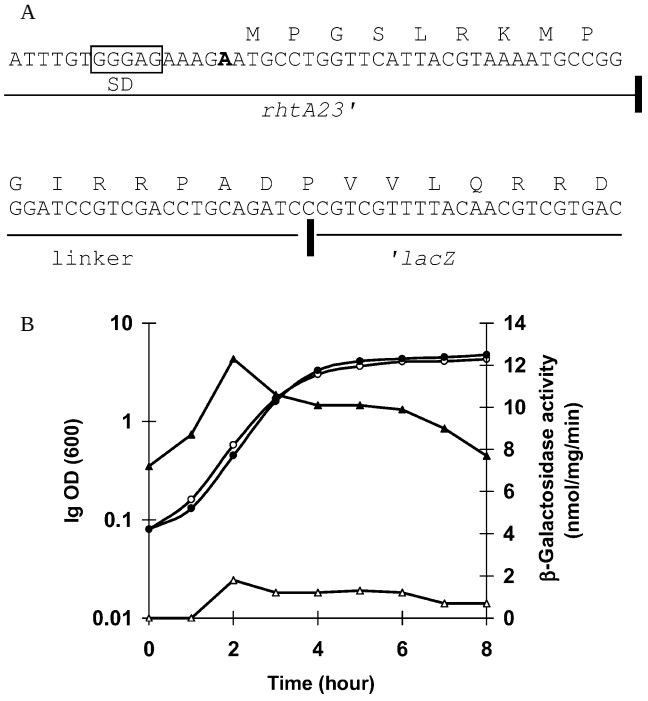

Fig. 3. Effect of *rhtA23* mutation on expression of *rhtA′–′lacZ* fusion. (A) The sequence of the *rhtA23′–′lacZ* fusion region (contained in pAZ2) is shown with the A-for-G substitution (marked in bold) that led to increased threonine and homoserine resistance. The predicted SD sequence is boxed. Bars divide the *rhtA*, linker and *lacZ* regions. (B) Expression of the *rhtA′–′lacZ* and *rhtA23′–′lacZ* translational fusions of strains TG1 (pAZ1) (open symbols) and TG1 (pAZ2) (closed symbols), respectively. Circles indicate lg OD_{600}, and triangles indicate β-galactosidase activity.

activity was assayed in the resulting strains, TG1 (pAZ1) and TG1 (pAZ2) (Fig. 3B). Both strains were found to have low β-galactosidase activity. These data agree with those of Lomovskaya et al. [24] where a low level expression of the ORF1 (rhtA)–lacZ fusion was found. However, β-galactosidase activity of the strain containing the pAZ2 plasmid was about 10 times higher than that of the strain containing the pAZ1 plasmid. Thus, the rhtA23 mutation enhances rhtA gene expression.

3.5. *Overexpression or inactivation of the* rhtA *gene affects* E. coli *cell resistance to amino acids and amino acid analogues*

To study the role that the *rhtA* gene plays in cell physiology, a chromosomal insertion mutation, *rhtA*::*kan*, was constructed as described in Materials and methods.

Inactivation of the *rhtA* gene did not affect the viability of the bacterium grown in rich or minimal media at different temperatures (results not shown). This agrees with data indicating that extended deletions of this chromosomal region are not lethal [28]. However, cells containing the *rhtA*$^+$ allele, the *rhtA23* mutation or the *rhtA*::*kan* insertion showed different susceptibility to some amino acids and amino acid analogues when grown in a minimal medium.

The *rhtA23* mutation provides cells with increased resistance to threonine, homoserine, some other amino acids, and to a variety of amino acid analogues: DL-hydroxynorvaline (threonine analogue), S(2-aminoethyl)-L-cysteine (lysine analogue), 4-aza-DL-leucine (leucine analogue), L-glutamic acid-γ-hydrazide (glutamine analogue) and 2,4-dehydro-DL-proline (proline analogue). A similar but less pronounced effect was observed in the case of the wild type *rhtA* gene cloned in a multicopy vector (Table 2). In contrast, we observed a slight increase in the susceptibility of the N99 *rhtA*::*kan* strain to some amino acids and amino acid analogues as compared to the N99 *rhtA*$^+$ strain (Table 2). These results were similar to the increased resistance or susceptibility to multiple drugs in the case of overexpression or inactivation, respectively, of genes encoding multidrug efflux pumps [27]. However, overexpression of the *rhtA* gene does not affect resistance of cells to chloramphenicol, ampicillin, tetracycline, rifampicin, polymyxin, acridine and sodium deoxycholate. In addition, overexpression of *rhtA* does not affect the ability of the cells to grow in hypertonic media (results not shown).

3.6. *Overexpression of the* rhtA *gene leads to decreased accumulation of homoserine and threonine in cells*

In light of the findings presented above we presumed that the increased resistance of *rhtA23* mutants to threonine and homoserine was a result of enhanced excretion of these amino acids. It is known that in the case of efflux pump gene overexpression, accumulation of the respective inhibitor in cells is decreased [27]. Therefore, we measured [^{3}H]homoserine and [^{3}H]threonine accumulation in *E. coli* N99 cells containing different *rhtA* alleles. As shown in Fig. 4A, at 8.5 min the accumulation level of homoserine in the cells of the *rhtA*$^+$ strain was about 5 times higher than in the isogenic *rhtA23* mutant. Similar results were also observed reproducibly for threonine (Fig. 4B).

Under the conditions of the experiment, protein synthesis was inhibited by chloramphenicol, and accumulation of the amino acids in cells was a result of the two simultaneous processes: amino acid entry into the cytoplasm, and their efflux from the cell. Any change in either process might influence the accumulation of amino acids. However, the *rhtA23* mutation did not affect homoserine transport into the cells, because the homoserine auxotrophic strain Gif102 [42] could grow at a low homoserine concentration (10 μg/ml) after the introduction of the *rhtA23* mutation. Moreover, a threonine auxotrophic derivative of N99 *rhtA23*, the N99 *rhtA23 thr*::Tn*10* strain (which was obtained by P1 transduction of the *thr*::Tn*10* mutation from the B-6105 strain) grew about equally well when supplied with L-alanyl-L-threonine dipeptide or L-threonine at a concentration of 0.1 mM (threonine concentration of 11.9 μg/ml) (results not shown). Meanwhile, amino acid transport-deficient mutants are known to exhibit the phenotype of a requirement for high amino acid concentrations in an appropriate aux-

Table 2
Effect of *rhtA* gene overexpression or inactivation on *E. coli* N99 resistance to amino acids and amino acid analogues

Substrate	Minimal inhibitory concentration (MIC) (mg/ml)			
	N99 *rhtA*$^+$	N99 *rhtA23*	N99 (pNPZ16)	N99 *rhtA*::*kan*
L-homoserine	0.75	>100	50	0.75
L-threonine	30	100	70	20
L-proline	1	5	3	1
L-lysine	60	70	n.d.[a]	60
L-asparagine	50	80	n.d.	50
L-serine	10	40	20	10
L-cysteine	2	6	n.d.	2
L-glutamate (Na salt)	10	30	n.d.	10
L-glutamine	75	90	n.d.	75
Glycine	15	20	n.d.	15
L-histidine	5	20	10	2.5
L-valine	0.001	0.003	n.d.	0.001
L-arginine	15	15	n.d.	15
L-aspartate (K salt)	60	60	n.d.	60
L-methionine	30	30	n.d.	30
L-phenylalanine	20	20	n.d.	20
L-tryptophane	15	15	n.d.	15
β-alanine	55	90	n.d.	45
S(2-aminoethyl)-L-cysteine	0.01	0.25	n.d.	0.01
4-aza-DL-leucine	0.05	0.5	0.25	0.025
2,4-dehydro-DL-proline	0.005	0.1	n.d.	0.005
L-glutamic acid-γ-hydrazide	0.05	0.25	n.d.	0.025
DL-hydroxynorvaline	0.1	1.0	0.2	0.05

[a]n.d., not determined.

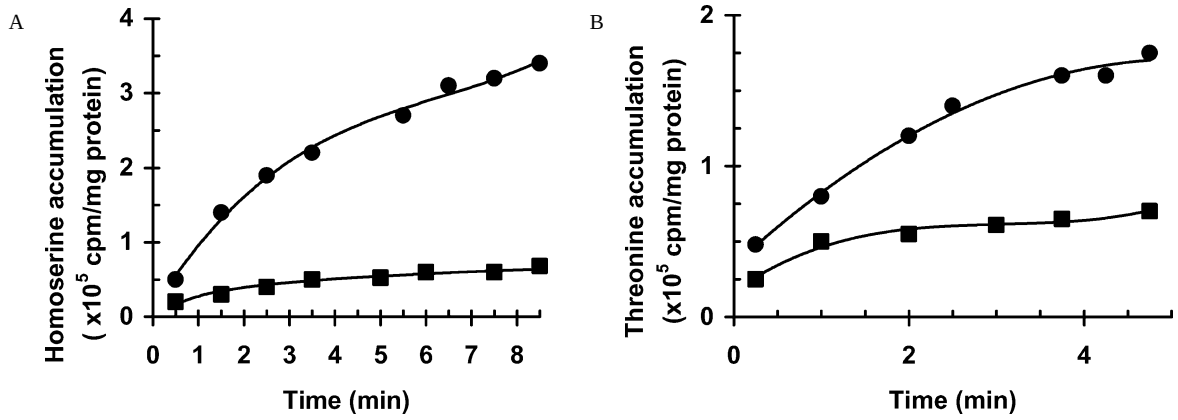

Fig. 4. Effect of the *rhtA23* mutation on homoserine and threonine accumulation in cells. (A) Accumulation of [^{3}H] homoserine in cells of strains *E. coli* N99 *rhtA*$^+$ (circles) and *E. coli* N99 *rhtA23* (squares). (B) Accumulation of [^{3}H] threonine in cells of strains *E. coli* N99 *rhtA*$^+$ (circles) and *E. coli* N99 *rhtA23* (squares).

otrophic background, and grow well at low concentrations of dipeptides containing the respective amino acid [29]. Dipeptides enter the cells by means of specific transport systems distinct from those of amino acids [32]. Consequently, the difference in homoserine and threonine accumulation between N99 *rhtA*$^+$ and N99 *rhtA23* strains is due to the increased efflux of these amino acids by the *rhtA23* mutants.

No pronounced effect of the *rhtA*::*kan* mutation on homoserine and threonine accumulation in cells was found in these experiments (data not shown). The latter result can be explained by the low expression level of the *rhtA* gene in the wild type strain, and by the presence of alternative efflux systems for threonine and homoserine in *E. coli* [51].

3.7. Overexpression of the rhtA *gene promotes excretion of threonine and homoserine*

Strain MG442 is a threonine producer in which threonine biosynthesis is released from feed-back regulation [12]. Its ability to produce threonine was further enhanced by introduction of the pVIC40 plasmid containing the *thrA442BC* operon in which the bifunctional enzyme aspartate kinase (EC 2.7.2.4)-homoserine dehydrogenase I (EC 1.1.1.3), encoded by the *thrA442* gene, is released from feedback inhibition by threonine [7]. This transformation produced strain MG442 (pVIC40). Strain NZ10 can produce homoserine due to the *thrB* mutation that impairs homoserine kinase

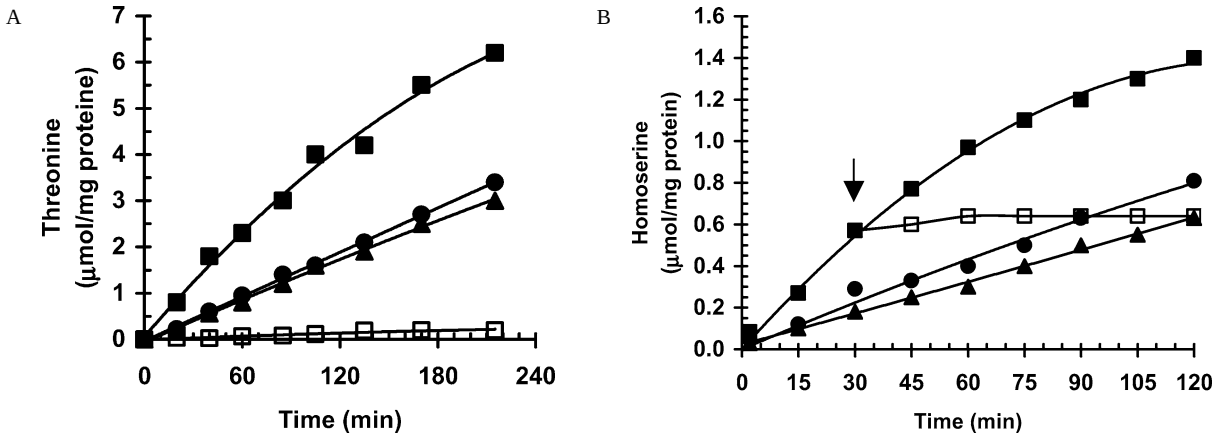

Fig. 5. Effect of *rhtA* gene overexpression or inactivation on the rate of threonine and homoserine excretion. (A) Effect of the different *rhtA* gene alleles on the rate of threonine excretion by the MG442 (pVIC40) strain. Threonine accumulation in medium by the strains: MG442 *rhtA*$^+$ (pVIC40) (circles), MG442 *rhtA23* (pVIC40) (squares) and MG442 *rhtA*::*kan* (pVIC40) (triangles). Open squares indicate threonine accumulation by strain MG442 *rhtA23* (pVIC40) with 20 μM CCCP added at time zero. (B) Effect of the different *rhtA* gene alleles on the rate of homoserine accumulation by the NZ10 (pAL4) strain. Homoserine accumulation in medium by strains NZ10 *rhtA*$^+$ (pAL4) (circles), NZ10 *rhtA23* (pAL4) (squares) and NZ10 *rhtA*::*kan* (pAL4) (triangles). Open squares show homoserine accumulation by strain NZ10 *rhtA23* (pAL4) after the addition of CCCP at the time indicated by the arrow.

(EC 2.7.1.39). Introduction of the pAL4 plasmid containing the wild type *thrA* gene improved its homoserine productivity [51].

In a short-term fermentation with MG442 (pVIC40), the *rhtA23* mutation enhanced the rate of threonine excretion. As shown in Fig. 5A, at 3 h the amount of threonine in the medium was about 2 times higher in the case of the *rhtA23* mutant than in the case of the isogenic *rhtA*$^+$ strain. Similarly, the NZ10 *rhtA23* (pAL4) strain excreted homoserine at a higher rate than the NZ10 (pAL4) strain (Fig. 5B). Reducing the electrochemical proton gradient across the cytoplasmic membrane by the addition of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) led to a drastic reduction in the rate of threonine and homoserine excretion by the respective *rhtA23* mutants (Fig. 5A and 5B).

The effect of the *rhtA23* mutation on threonine production was not caused by its influence on threonine biosynthesis and accumulation in cells, because the *rhtA23* mutation decreased the intracellular pool of this amino acid. The pool of threonine in the cells of the MG442 *rhtA23* (pVIC40) strain was 30 ± 8 nmol/mg (DW), and that in the cells of the MG442 *rhtA*$^+$ (pVIC40) was 187 ± 53 nmol/mg (DW).

Taken together, the above data strongly suggested the involvement of RhtA in the efflux of threonine and homoserine driven by the proton motive force.

The *rhtA*::*kan* mutation had only a small effect on threonine and homoserine accumalation (Fig. 5A and 5B), which may be explained by the presence of alternative efflux systems for threonine and homoserine [51].

3.8. Overexpression of the rhtA *gene improves amino acid productivity of the respective producing strains*

As shown above, the *rhtA23* mutation increased threonine and homoserine accumulation by the respective producing strains. In pH-controlled fermenter experiments strain MG442 *rhtA*$^+$ (pVIC40) accumulated 18.4 ± 0.9 g/l threonine and strain MG442 *rhtA23* (pVIC40)—36.3 ± 3.2 g/l threonine. The optical densities of cultures in these fermentations were about the same. Homoserine accumulation by strains NZ10 *rhtA*$^+$ (pAL4) and NZ10 *rhtA23* (pAL4) under this condition was, accordingly, 9.0 ± 0.6 g/l and 56.0 ± 2.6 g/l. This marked difference seemed to be dependent on the strong inhibitory effect of homoserine, which accumulated in *rhtA*$^+$ cells, upon their metabolism and growth (see Table 1). The optical density of strain NZ10 *rhtA*$^+$ (pAL4) culture by the end of fermentation was 1.6-fold lower than that of strain NZ10 *rhtA23* (pAL4) (data not shown). Thus, enhancement of excretion caused by overexpression of the *rhtA* gene improved threonine and homoserine productivity of the respective producing strains.

Besides threonine and homoserine, the *rhtA23* mutation conferred upon cells increased resistance to several other amino acids, specifically, proline, lysine and their analogues (Table 1). Therefore, the effect of this mutation on productivity of the respective producing strains was tested by tube fermentation. Strain VL2151 can accumulate proline in a culture broth [22]. Introduction of the *rhtA23* mutation produced strain VL2151 *rhtA23*. When cultured in fermentation medium strains VL2151 (*rhtA*$^+$) and VL2151 *rhtA23* accumulated 1.7 ± 0.1 g/l and 2.4 ± 0.1 g/l proline, accordingly. Strain VL614 accumulated some amounts of lysine [22]. Introduction of the *rhtA23* mutation produced strain VL614 *rhtA23*. When cultured in fermentation medium containing limiting concentrations of homoserine these strains accumulated 2.7 ± 0.1 g/l and 3.7 ± 0.2 g/l lysine, accordingly. The optical densities of the cultures by the end of fermentation for each pair *rhtA*$^+$ and *rhtA23* of the strains were practically the same (data not shown). Thus, the *rhtA23* mutation improved lysine and proline productivity of the respective producing strains.

3.9. The RhtA protein belongs to a vast family of transporters

Data bank search revealed about 250 protein homologues to RhtA. Among them were: YdeD from *E. coli*, involved in efflux of metabolites of the cysteine pathway [6]; PecM from *Erwinia chrysanthemi* involved in the efflux of the blue pigment indigoidine [34]; YddG from *Salmonella enterica* sv. Typhimurium involved in the efflux of methyl viologen [37]; triose phosphate/phosphate, phosphoenolpyruvate/phosphate, and glucose-6-phosphate translocators from plant chloroplasts, nucleotide sugar transporters of the Golgi apparatus and the endoplasmic reticulum of eukaryotic cells (for example, P52177, P52178, O64909, Q61420, and T15823 in Fig. 6). A small fraction of these proteins contained about 150 amino acid residues that are homologues to the N-terminal part of RhtA (e.g., AQ1741 from *Aqifex aeolicus* in Fig. 6), or the C-terminal part of RhtA (e.g., ORFB from *E. coli* in Fig. 6). However, most of the proteins have a similar size (about 300 amino acid residues), a similar hydropathy profile, and contain 10 predicted transmembrane segments. Based on these similarities, we consider these proteins as a vast family of transporters. The members of this family, however, have a wide variety of a primary structure. We also included in the family some proteins that differed in their size from the majority. These differences are caused by the presence of a leader peptide at the N-terminus that is deleted from the mature protein (e.g., chloroplast transporters, P52177, P52178 in Fig. 6), or a histidine tag at the C-terminus, obviously needed for binding of nickel, in CnrT protein from *Ralstonia* sp. [10]. For some family members these differences may be caused by inaccurate definition of the edges of coding sequences. For example, the hypothetical YSO2_ACIAM protein of archaebacterium *Acidianus* (*Desulfurolobus*) *ambivalens* (P29086 in Fig. 6) has a putative size of only 253 amino acid residues and contains 9 predicted transmembrane segments. However, the prolongation of the coding sequence by 31 triplets upstream of the putative start codon results in an increase in the protein length, so that the 10th transmembrane segment and several conserved residues in the sequence are added.

The majority of the RhtA homologues that we found belong to the membrane protein family Pfam00892 (DUF6) [4]. In addition, most of these proteins were included in the cluster of orthologous group (COG) 0697 as permeases of the drug/metabolite transporter (DMT) superfamily [41] and in the drug/metabolite transporter (DMT) superfamily of transporter proteins [17].

Some bacterial genomes contain multiple RhtA paralogues. For example, at least 10 RhtA paralogues are present in the genome of *E. coli*, and at least 14 RhtA paralogues are present in the genome of *B. subtilis* (Fig. 6).

Phylogenetic analysis showed that several clusters of the homologues (that may be considered as different subfamilies) include proteins from bacteria, archaea and eukarya (Fig. 6). These data suggest that a common ancestor of the living organisms contained several RhtA paralogues, proteins with 10 transmembrane segments.

4. Discussion

In this study, we identified and characterized the *rhtA* gene, which encodes a membrane protein involved in the efflux of threonine and homoserine in *E. coli*. This protein belongs to a vast family of transporters. The *rhtA23* mutation is an A-for-G substitution at position −1 in relation to the ATG start codon of the gene (Fig. 3A). It is known that the nucleotide composition upstream or downstream of the SD region influences mRNA translational efficiency [25]. A 20-fold range in the *lacZ* expression levels has been found, depending on the nature of the three nucleotides preceding the start codon [16]. Accordingly, we showed that the *rhtA23* mutation increased at least 10-fold the expression level of *rhtA′—′lacZ* translation fusion (Fig. 3B), which indicates enhanced *rhtA* gene expression.

rhtA gene overexpression conferred upon cells increased resistance not only to threonine and homoserine, but also to a variety of other amino acids and amino acid analogues (Table 1). This effect resembles the overexpression of genes encoding multidrug efflux pumps [27], and indicates a broad specificity of the RhtA transporter. Of course, different degrees of susceptibility, as reflected by MICs, are not necessarily a reflection of the specificity of the pump, since other factors may also play a role. However, in this study, we showed that in addition to threonine and homoserine RhtA overexpression had some positive effects on production of lysine and proline, obviously due to the enhanced excretion of these amino acids. Thus, proline and lysine are also among the substrates of the RhtA transporter.

The multiple effect of RhtA overexpression might also be related to its additional regulatory function. In this context, the RhtA homologue, the PecM protein of *E. chrysanthemi*, involved in efflux of the blue pigment indigoidine, was also found to be a component of the PecS/PecM regulatory couple responsible for the expression of many virulence genes [33,34].

The hydropathy profile and the predicted transmembrane segments of RhtA are very similar to those of the YdeD protein (Fig. 2A) that obviously reflects their structural homology and related function [23]. YdeD is involved in efflux of metabolites of the cysteine pathway [6]. It is interesting to note that the *rhtA23* mutation conferred upon cells increased resistance to cysteine (Table 1). On the other hand, we found that YdeD overexpression increased cell resistance not only to cysteine, but also to threonine (unpublished data). It is therefore possible that the specificity of YdeD is not restricted to cysteine-related compounds.

The inactivation of the *rhtA* gene had a rather slight—if any—effect on threonine and homoserine resistance and on accumulation of threonine and homoserine in the cells and in the medium. The small magnitude of this effect can

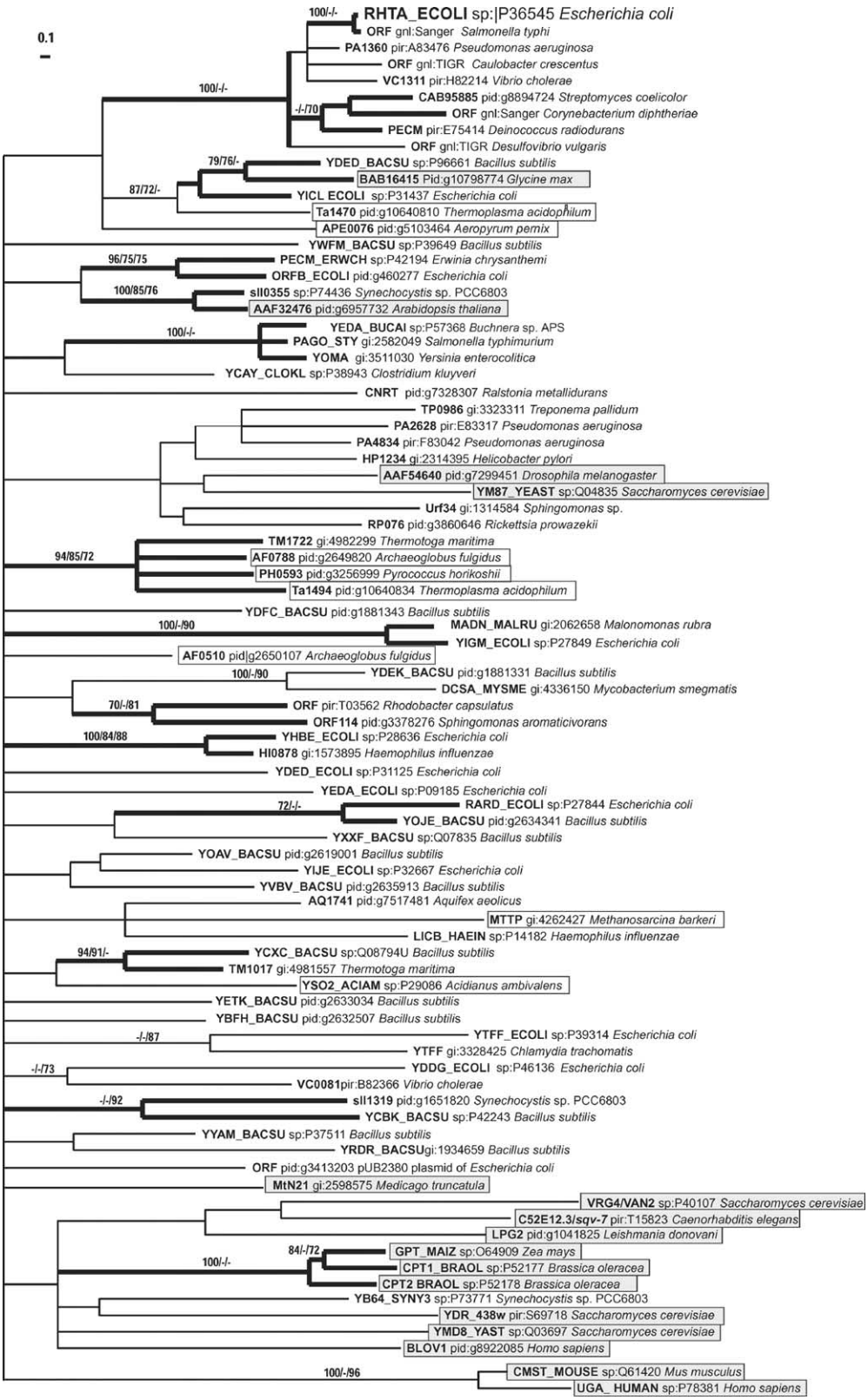

Fig. 6. Phylogenetic tree of some of the RhtA family member proteins. The selected protein set reflects the taxonomic and structural diversity of this family and includes all found paralogues from genomes of *E. coli* and *B. subtilis*. Numbers above the branches are bootstrap percentage support of 100 replicates for the most parsimonious trees, 1000 replicates for neighbour-joining trees or 50 000 puzzling in maximum likelihood trees, respectively. Branches with greater than 70% support are only presented. The branches that were found in a major rule consensus tree for each of the three analysis methods are shown as thick lines. The consensus tree has a length of 13 966 steps, CI = 0.274; $\log L = -51990.42$; branches' lengths are calculated as the most likelihood. Proteins of archaea are in the open boxes, proteins of eukarya are in the gray boxes.

be explained by the presence of alternative efflux systems for threonine and homoserine encoded by the *rhtB* and *rhtC* genes [51] and possibly by other genes. Excretion of the same substance by several systems is well known for multidrug efflux pumps [27]. We suggest that several amino acid efflux systems might have different functions in a bacterial cell, and/or are induced under different conditions.

The physiological significance of amino acid excretion in bacteria is not clear. The existence of transporters conducting the excretion of amino acids may be needed for maintenance of balanced intracellular pools of amino acids. Our experiments revealed that nearly all of the natural L-amino acids at a high concentration inhibit the growth of *E. coli* in minimal media (Table 1). An inhibitory effect of the amino acids, except for glycine, tryptophan and phenylalanine, was not observed in rich media or in minimal media containing the other 19 natural L-amino acids, each at a concentration of 0.1 mM (unpublished data). Therefore, with the exception of aromatic amino acids and glycine, amino acid-dependent growth inhibition seems to be caused by the suppression of metabolism of other amino acids. Amino acid efflux may consequently prevent swelling of amino acid pools to inhibiting levels under some conditions and may play a regulatory role in bacterial amino acid metabolism.

Moreover, the excretion of amino acids or of their derivatives might be necessary for the interaction between bacterial symbionts and their hosts, and for cell-to-cell communication.

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References

[1] J. Adachi, M. Hasegawa, Instability of quartet analyses of molecular sequence data by the maximum likelihood method: the Cetacea/Artiodactyla relationships, Mol. Phylogenet. Evol. 6 (1996) 72–76.

[2] V.V. Aleshin, N.P. Zakataeva, V.A. Livshits, A new family of amino acid efflux proteins, Trends Biochem. Sci. 24 (1999) 133–135.

[3] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: A new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[4] A. Bateman, E. Birney, R. Durbin, S.R. Eddy, K.L. Howe, E.L.L. Sonnhammer, The Pfam protein family database, Nucleic Acids Res. 28 (2000) 263–266.

[5] A. Bellmann, M. Vrljić, M. Pátek, H. Sahm, R. Krämer, L. Eggeling, Expression control and specificity of the basic amino acid exporter LysE of *Corynebacterium glutamicum*, Microbiology 147 (2001) 1765–1774.

[6] T. Daßler, T. Maier, C. Winterhalter, A. Böck, Identification of a major facilitator protein from *Escherichia coli* involved in efflux of metabolites of the cysteine pathway, Mol. Microbiol. 36 (2000) 1101–1112.

[7] V.G. Debabov, J.I. Kozlov, E.M. Khurges, V.A. Livshits, N.I. Zhdanova, M.M. Gusyatiner, A.K. Sokolov, T.A. Bachina, Bacterial strain of *Escherichia coli* VNII GENETIKA 472T23 as the producer of L-threonine, United States patent 5,631,157, Ajinomoto, Tokio, Japan, 1997.

[8] H. Ebbighausen, B. Weil, R. Krämer, Isoleucine excretion in *Corynebacterium glutamicum*: Evidence for a specific efflux carrier system, Appl. Microbiol. Biotechnol. 31 (1989) 184–190.

[9] J. Felsenstein, PHYLIP—Phylogeny Inference Package, version 3.5, University of Washington, Seattle, 1993.

[10] G. Grass, C. Große, D.H. Nies, Regulation of the *cnr* cobalt and nickel resistance determinant from *Ralstonia* sp. Strain CH34, J. Bacteriol. 182 (2000) 1390–1398.

[11] E.A. Groisman, M.J. Casadaban, Mini-Mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing, J. Bacteriol. 168 (1986) 357–364.

[12] M.M. Gusyatiner, N.I. Zhdanova, V.A. Livshits, G.G. Zaigraeva, R.S. Shakulov, Investigation of the *relA* gene function in the expression of amino acid operons. II. Effect of the *relA* gene allelic state on the overproduction of threonine by an *Escherichia coli* K-12 mutant resistant to β-hydroxynorvaline, Genetika 14 (1978) 957–968 (in Russian).

[13] H. Hama, T. Kayahara, M. Tsuda, T. Tsuchiya, Inhibition of homoserine dehydrogenase I by L-serine in *Escherichia coli*, J. Biochem. (Tokyo) 109 (1991) 604–608.

[14] C.L. Harris, Cysteine and growth inhibition of *Escherichia coli*: Threonine deaminase as the target enzyme, J. Bacteriol. 145 (1981) 1031–1035.

[15] Y. Hirose, H. Okada, Microbial production of amino acids, in: H.J. Peppler, D. Perlman (Eds.), Microbial Technology, Vol. 1, Academic Press, New York, 1979, pp. 211–244.

[16] A. Hui, J. Hayflick, K. Dinkelspiel, H.A. de Boer, Mutagenesis of the three bases preceding the start codon of the β-galactosidase mRNA and its effect on translation in *Escherichia coli*, EMBO J. 3 (1984) 623–629.

[17] D.L. Jack, N.M. Yang, M.H. Saier Jr., The drug/metabolite transporter superfamily, Eur. J. Biochem. 268 (2001) 3620–3639.

[18] A.M. Kotre, S.J. Sullivan, M.A. Savageau, Metabolic regulation by homoserine in *Escherichia coli* B/r, J. Bacteriol. 116 (1973) 663–672.

[19] R. Krämer, Systems and mechanisms of amino acid uptake and excretion in prokaryotes, Arch. Microbiol. 162 (1994) 1–13.

[20] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.

[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[22] V.A. Livshits, N.P. Zakataeva, K. Nakanishi, V.V. Aleshin, P.V. Troshin, I.L. Tokmakova, *Escherichia coli* DNA fragment determining enhanced production of amino acids (variants) and method of amino acid producing, Russian patent No. 2175351, GNII Genetika, Moscow, Russia, 2001.

[23] J.S. Lolkema, D.-J. Slotboom, Hydropathy profile aligment: A tool to search for structural homologues of membrane proteins, FEMS Microbiol. Rev. 22 (1998) 305–322.

[24] O.L. Lomovskaya, J.P. Kidwell, A. Matin, Characterization of the σ^{38}-dependent expression of a core *Escherichia coli* starvation gene, *pexB*, J. Bacteriol. 176 (1994) 3928–3935.

[25] S.C. Makrides, Strategies for achiving high-level expression of genes in *Escherichia coli*, Microbiol. Rev. 60 (1996) 512–538.

[26] J.H. Miller (Ed.), Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, New York, 1972.

[27] H. Nikaido, Multidrug efflux pumps of gram-negative bacteria, J. Bacteriol. 178 (1996) 5853–5859.

[28] T. Nochno, T. Saito, J.-S. Hong, Cloning and complete nucleotide sequence of the *Escherichia coli* glutamine permease operon (*glnHPQ*), Mol. Gen. Genet. 205 (1986) 260–269.

[29] K. Okamoto, K. Kino, M. Ikeda, Hyperproduction of L-threonine by *Escherichia coli* mutant with impaired L-threonine uptake, Biosci. Biotech. Biochem. 61 (1997) 1877–1882.

[30] R.D.M. Page, TREEVIEW: An application to display phylogenetic trees on personal computers, Comp. Appl. Biosci. 12 (1996) 357–358.

[31] B. Parker, M.G. Marinus, A simple and rapid method to obtain substitution mutations in *Escherichia coli*: Isolation of a *dam* deletion/insertion mutation, Gene 73 (1988) 531–535.

[32] J.W. Payne, M.W. Smith, Peptide transport by microorganisms, Adv. Microb. Physiol. 36 (1994) 1–80.

[33] T. Praillet, S. Reverchon, J. Robert-Baudouy, W. Nasser, The PecM protein is necessary for the DNA-binding capacity of the PecS repressor, one of the regulators of virulence-factor synthesis in *Erwinia chrysanthemi*, FEMS Microbiol. Lett. 154 (1997) 265–270.

[34] C. Rouanet, W. Nasser, The PecM protein of the phytopathogenic bacterium *Erwinia chrysanthemi*, membrane topology and possible involvement in the efflux of the blue pigment indigoidine, J. Mol. Microbiol. Biotechnol. 3 (2001) 309–318.

[35] M.H. Saier Jr., I.T., Paulsen, M.K. Sliwinski, S.S. Pao, R.A. Skurrey, H. Nikaido, Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria, FASEB J. 12 (1998) 265–274.

[36] J. Sambrook, E.F. Fritsch, T. Maniatis (Eds.), Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.

[37] C.A. Santiviago, J.A. Fuentes, S.M. Bueno, A.N. Trombert, A.A. Hildago, L.T. Socias, P. Youderian, G.C. Mora, The *Salmonella enterica* sv. Typhimurium *smvA*, *yddG*, and *ompD* (porin) genes are required for the efficient efflux of methyl viologen, Mol. Microbiol. 46 (2002) 687–698.

[38] P. Simic, H. Sahm, L. Eggeling, L-Threonine export: Use of peptides to identify a new translocator from *Corynebacterium glutamicum*, J. Bacteriol. 183 (2001) 5317–5324.

[39] K. Strimmer, A. von Haeseler, Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies, Mol. Biol. Evol. 24 (1996) 964–969.

[40] S. Tabor, C.C. Richardson, A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes, Proc. Natl. Acad. Sci. USA 82 (1985) 1074–1078.

[41] R.L. Tatusov, D.N. Natale, I.V. Garkavtsev, T.A. Tatusova, U.T. Shankavaram, B.S. Rao, B. Kiryutin, M.Y. Galperin, N.D. Fedorova, E.V. Koonin, The COG database: New developments in phylogenetic classification of proteins from complete genomes, Nucleic Acids Res. 28 (2001) 263–266.

[42] J. Theze, I. Saint-Girons, Threonine locus of *Escherichia coli* K-12: Genetic structure and evidence for an operon, J. Bacteriol. 118 (1974) 990–998.

[43] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.

[44] G.E. Tusnády, I. Simon, Principles governing amino acid composition of integral membrane proteins: Application to topology prediction, J. Mol. Biol. 283 (1998) 489–506.

[45] H.E. Umbarger, Biosynthesis of the branched-chain amino acids, in: F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaecher, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella typhymurium*: Cellular and Molecular Biology, Vol. 1, American Society for Microbiology Press, Washington, 1996, pp. 442–457.

[46] H.E. Umbarger, Metabolic analogues as genetic and biochemical probes, Adv. Genet. 16 (1971) 119–140.

[47] J. Vieira, J. Messing, New pUC-derived cloning vectors with different selectable markers and DNA replication origins, Gene 100 (1991) 189–194.

[48] M. Vrljic, H. Sahm, L. Eggeling, A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*, Mol. Microbiol. 22 (1996) 815–826.

[49] B. Wallace, Y.-J. Yang, J. Hong, D. Lum, Cloning and sequencing of a gene encoding a glutamate and aspartate carrier of *Escherichia coli* K-12, J. Bacteriol. 172 (1990) 3214–3220.

[50] M.-R. Yen, Y.-H. Tseng, P. Simic, H. Sahm, L. Eggeling, M.H. Saier Jr., The ubiquitous ThrE family of putative transmembrane amino acid efflux transporters, Res. Microbiol. 153 (2002) 19–25.

[51] N.P. Zakataeva, V.V. Aleshin, I.L. Tokmakova, P.V. Troshin, V.A. Livshits, The novel transmembrane *Escherichia coli* proteins involved in the amino acid efflux, FEBS Lett. 452 (1999) 228–232.