Acinetobacter calcoaceticus encoded mutarotase: nucleotide sequence analysis of the gene and characterization of its secretion in *Escherichia coli*

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ABSTRACT

The nucleotide sequence of the mutarotase gene from Acinetobacter calcoaceticus has been determined. It reveals an open reading frame of 381 amino acids. The codon usage of A. calcoaceticus for this gene is similar to E. coli except for the amino acids Leu, Ala, Glu, and Arg where major differences exist. This did not interfere drastically with high level expression in E. coli. The regulatory sequences for the initiation of translation are similar to the ones described for E. coli. The N-terminal 20 amino acids, which are not found in the mature enzyme, show homology to signal sequences of exported proteins. In A. calcoaceticus and E. coli mutarotase is specifically secreted into the periplasmic space. Processing of the signal sequence occurs at identical sites in both organisms The mature mutarotase consists of 361 amino acids and has a calculated molecular weight of 38457 Da. Expression of mutarotase at a high level in a recombinant E. coli destabilizes the outer membrane. This results in coordinated leakage of mutarotase and ß-lactamase into the culture broth.

INTRODUCTION

Mutarotase (E.C.5.1.3.3.) is an accelerator of the anomeric interconversion of D-glucoses and other aldoses (1,2). Despite its frequent diagnostic application in quantitative assays of D-glucose in body fluids (3-5) and its wide distribution among living organisms ranging from microorganisms (6) to mammals (7-9) only very limited knowledge exists regarding its structure and function.

Recently, we have reported the purification and partial amino acid sequence analysis of mutarotase from Acinetobacter calcoaceticus (45). The gene encoding mutarotase (mro) was isolated from A. calcoaceticus, transferred into E. coli, and expressed under transcriptional control of the phage lambda promotor P_L. The mutarotase activity expressed by the recombinant E. coli was located in the culture medium. Excretion of proteins across both membrans of E. coli has been reported only for hemolysin (10) and other endotoxins (11,12). Non-specific leakage through the outer membrane has been described for the expression of penicillinase from alkalo-

philic Bacillus sp. in E. coli (13).

In this article we describe the nucleotide sequence analysis of the mro gene from A. calcoaceticus as the first sequenced gene from this organism and characterize a leader peptide active in both A. calcoaceticus and E. coli. The excretion of mutarotase into the culture medium of E. coli was found to be the result of specific export into the periplasm and non-specific leakage through the outer membrane.

MATERIALS AND METHODS

Bacterial strains, chemicals and general methods

E. coli R/RI (14) was generally used as a host for transformations. E. coli WH1372 is E. coli K12ΔH1Δtrp (15) transformed with pWH1372 which contains the mutarotase gene cloned between the filled in EcoRI site and the SalI site of pWH305 (26). This construction places mutarotase expression under transcriptional control of the phage lambda promotor P_L (45). E. coli WH602 is E. coli K12ΔH1Δtrp (15) transformed with pWH602 (16). pUR250 (17) was used to subclone a RsaI fragment to facilitate sequencing. Growth of bacteria and expression and secretion experiments were done in L-broth (18).

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and calf intestine phosphatase were purchased from Boehringer, Mannheim, and used as recommended by the producer. Polyacrylamide and agarose gel electrophoresis and SDS polyacrylamide gel electrophoresis was done as described previously (16). ^{32}P and $\alpha^{32}P$dATP were purchased from Amersham, Braunschweig. $\gamma^{32}P$ATP was synthezised from ^{32}P and ADP as described previously (19). General chemicals were purchased from Merck, Darmstadt.

Nucleotide sequencing

Determination of nucleotide sequences was done by the chemical method (20) and by the chain termination method (21). For chemical sequencing a kit obtained from NEN, Dreieich, was used. For sequencing with the chain termination method a kit purchased from Amersham, Braunschweig, was used. All procedures were done essentially as described. The sequencing gels were prepared and run as described (22).

Mutarotase assays

One unit of mutarotase activity is defined as the amount of enzyme converting one µMole α-D-glucose more to ß-D-glucose in one min at 22°C, pH 7.2, than does the naturally occuring mutarotation. It was measured as described previously (45). The cell associated activity was determined af-

ter pelleting and washing the cells once in 50 mM Tris HCl pH 8.0, 0.1 mM EDTA as described before. Mutarotase activity in the culture medium was measured directly in the supernatant after pelleting the cells. Mutarotase activity in the periplasmic space was measured after osmotic shock or treatment of the cells with triton X100.

ß-lactamase assays

ß-lactamase activity was assayed essentially as described (23). 1 mg nitrocefin was dissolved in 1 ml DMSO. 50 mM Tris HCl pH 7.0 was added so that the absorption at 390 nm was between 0.9 and 1.5. The ß-lactamase containing cell extracts and media were prepared as described for the mutarotase assays above. Samples of these were added to the nitrocefin solution to give a decrease of absorption between 0.01 and 0.04 per minute at 390 nm. ß-lactamase activity was expressed as ΔA_{390}/min mol.

RESULTS

Nucleotide sequence analysis of the mro gene

The sequencing strategy for the mro gene is displayed in Figure 1. Except for the RsaI fragment spanning from position 868 to 1119 it makes use of rare rstriction sites. The 251 bp RsaI fragment was subcloned into the single HincII site of pUR250 (17) to yield pWH1306, which was then used for sequencing. For the most part the sequences of both strands were determined independently. When this was difficult due to the lack of suitable restriction sites and the amino acid sequence of that part had been determined previously (45), no attempt was made to analyze the missing DNA strand (see Figure 1).

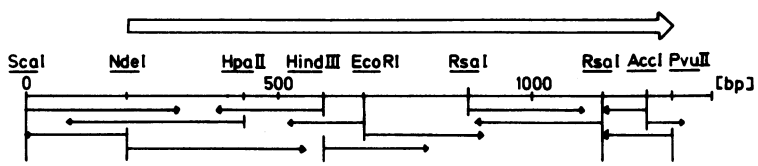

Fig. 1: Sequencing strategy of the mro gene
The DNA is depicted as a line and the numbers indicate the length of the DNA in base pairs starting from the ScaI site at the leftmost end of this drawing. Restriction sites used for sequencing experiments are indicated. Each sequencing experiment is represented by an arrow indicating the direction and length of the sequence determined. The open arrow on the top of the figure gives the location of the mutarotase reading frame with respect to the DNA depicted.

Fig. 2: Nucleotide sequence of the A. calcoaceticus encoded mutarotase gene

1320 positions of the nucleotide sequence are shown beginning at the ScaI site. The non coding sequence is shown double stranded while for the open reading frame only the coding strand is displayed. The deduced amino acid sequence of mutarotase is shown above the nucleotide sequence. The first 20 amino acids constitute a leader sequence, the next 27 amino acids and 76 amino acids from the C-terminus agree with the partially determined primary structure of mutarotase prepared from A. calcoaceticus.

Figure 2 shows the nucleotide sequence of 1320 bp including the mro gene from A. calcoaceticus beginning at the ScaI site. It was analyzed for restriction endonuclease recognition sites. The endonucleases and the positions of their respective recognition sites are listed in Table I.

An open reading frame starts at the ATG codon at position 121 and ends at the termination codon TAG at position 1264. It encodes 381 amino acids which are also depicted in Figure 2. Comparison of this primary structure with the N-terminal amino acid sequence of mutarotase (45) reveals, that the first 20 amino acids are not found in the mature enzyme. This result implies that mutarotase is synthezised as a precurser and afterwards processed to the mature enzyme. The deduced C-terminal amino acid sequence agrees with the one determined for mutarotase from A. calcoaceticus. The function of the N-terminal leader peptide of 20 amino acids will be analyzed below.

The primary structure of the mro gene determined here agrees very well with the partial primary structure of mutarotase. With one exception the N-terminal 27 and the C-terminal 76 amino acids are found to be identical in

Table 1: Cleavage sites of restriction endonucleases in the mro gene
The TaqI- and the ClaI sites marked * are protected by dam-methylation (27).

Restrictionendonuclease	Position
AccI	1073, 1196
AflII	588
AflIII	533
AhaIII	55, 1205
AluI	156, 453, 581, 1004, 1305
AvaII	492, 1033
BalI	218
BbvI	177, 1002, 1306
BclI	701
BstEII	818
CfrI	218
ClaI	18*
DdeI	4, 455, 1302, 1318
EcoRI	672
EcoRII, BstNI	519
Fnu4HI	177, 511, 1002, 1306
FokI	85, 886
HaeI	218, 934
HaeIII, PalI	219, 807, 935
HgaI	1063, 1137
HincII	593, 717
HindIII	580
HinfI	209, 259, 308, 1178, 1298
HpaI	593
HpaII, MspI	431, 625
HphI	567, 846
MaeI	780, 1143
MnlI	51, 476, 554, 1276
NciI	492, 624
NdeI	199
NlaIII	119, 242, 786, 1037
NspBII	1304
PvuII	1304
RsaI	205, 792, 868, 1119
Sau3A, MboI, DpnI	17, 231, 612, 647, 663, 702, 783, 825, 1017, 1083
Sau96I	492, 1033
SciNI, HhaI, CfoI	1108
ScrFI	519, 624
SfaNI	315, 887
SspI	1098
TaqI	19*, 478, 914, 1127
Tth111II	326, 536, 1131
XmnI	390

both analyses. (45). This result is the final proof that the mro gene isolated from A. calcoaceticus indeed encodes the mutarotase isolated from the same organism before and that the reading frame in the nucleotide sequence in Figure 2 is correctly identified. The only disagreement regards the amino acid at position seven of the mature protein which was Ser in the E. coli derived product and Pro in the A. calcoaceticus derived protein. The nucleotide sequence predicts a Ser at this position and we assume that the Pro codon CCA was mutated to the Ser codon TCA during the cloning procedures (see Figure 2).

The molecular weight calculated from the nucleotide sequence of the mro gene for the precurser is 40,444 Da, when the N-terminal Met is not consi-

Table 2: Codon usage and amino acid composition of mutarotase

amino acid	codon	number of codons used in the mutarotase gene		amino acid	codon	number of codons used in the mutarotase gene	
Phe	TTT	13	16	Ala	GCT	6	
	TTC	3			GCC	4	25
Leu	TTA	16			GCA	14	
	TTG	3			GCG	1	
	CTT	4		Tyr	TAT	14	16
	CTC	5	33		TAC	2	
	CTA	3					
	CTG	2		His	CAT	5	6
					CAC	1	
Ile	ATT	12					
	ATC	4	17	Gln	CAA	22	25
	ATA	1			CAG	3	
Val	GTT	13		Asn	AAT	23	34
	GTC	10	33		AAC	11	
	GTA	6					
	GTG	4		Lys	AAA	20	26
					AAG	6	
Ser	TCT	7					
	TCC	-		Asp	GAT	16	20
	TCA	5	24		GAC	4	
	TCG	1					
	AGT	5		Glu	GAA	9	9
	AGC	6			GAG	-	
Pro	CCT	4		Arg	CGT	3	
	CCC	4	18		CGC	-	
	CCA	7			CGA	2	8
	CCG	3			CGG	3	
					AGA	-	
Thr	ACT	8			AGG	-	
	ACC	9	28				
	ACA	6		Gly	GGT	14	
	ACG	5			GGC	12	35
					GGA	8	
					GGG	1	

dered. The molecular weight of the mature protein is 38,457 Da. This is in excellent agreement with the value of 40 kDa determined by denaturing gel electrophoresis (45).

As far as we know mro is the first gene from Acinetobacter which has been isolated and sequenced. It is therefore interesting to compare its codon usage to the one in other organisms (24). Table II lists the codon usage in the mro gene and gives the amino acid composition of mutarotase. Most of the amino acids show codon preferences similar to the rarely expressed genes in E. coli (25). The amino acids Leu, Ala, Gln, and Arg exhibit codon preferences different from the ones found in E. coli genes. The codons TTA (Leu), GCA (Ala), CAA (Gln), and CGG (Arg) are more frequently used in A. calcoaceticus than in E. coli. CGC (Arg), which is the most frequently used codon in the weakly expressed E. coli genes, is not found in the mutarotase gene. This result may explain the observation, that the mro gene cloned under transcriptional control of the phage lambda promotor

Table 3: Comparison of procaryotic signal sequences

protein	charged segment	hydrophobic segment	cleavage site	Reference
mutarotase	MetLysLys	LeuAlaIleLeuGlyValThrValTyrSerPheAlaGlnLeuAlaAsn	Ala-Ala	
fd, minor coat protein	MetLysLys	LeuLeuPheAlaIleProLeuValValProPheTyrSerHis	Ser-Ala	30
alkaline phosphatase	MetLys	GlnSerThrIleAlaLeuAlaLeuLeuProLeuLeuPheThrProValThrLys	Ala-Arg	31
histidine binding protein of Salmonella typhimurium	MetLysLys	LeuAlaLeuSerLeuSerLeuValLeuAlaPheSerSerAlaThrAlaAlaPhe	Ala-Ala	32
lysine-arginine-ornithine-binding protein of S. typhimurium	MetLysLys	ThrValLeuAlaLeuSerLeuLeuIleGlyLeuGlyAlaThrAlaAlaSerTyr	Ala-Ala	32
omp C				
B-lactamase	MetPheLys	ThrThrLeuCysAlaLeuLueIleThrAlaSerCysSerThrPhe	Ala-Ala	33
omp A	MetLysLys	ThrAlaIleAlaIleAlaValAlaLeuAlaGlyPheAlaThrValAlaGln	Ala-Ala	34
heat labile toxin (B-subunit)	MetAsnLysValLys	CysTyrValLeuPheThrAlaLeuLeuSerSerLeuTyrAlaHis	Gly-Ala	11
heat labile toxin (A-subunit)	MetLys	AsnIleThrPheIlePhePheIleLeuLeuAlaSerProLeuTyr	Ala-Asn	35

P_L is not expressed as efficiently as other genes (26).

The amino acid composition of mutarotase depicted in Table II indicates that no cysteine occurs in this polypeptide. This may be the reason for the unusual stability of this protein. Furthermore, the basic nature of mutarotase noted before (45) is confirmed by the result that 34 arginin and lysin residues and only 28 glutamic acid and aspartic acid residues are found in mutarotase. When compared to the average amino acid composition of E. coli proteins the lack of acidic residues is due to the underrepresentation of glutamic acid. On the other band, the corresponding neutral residues asparagine and glutamine are overrepresented. The remaining amino acids are represented in the average amount (28).

Analysis of the leader peptide

The sequence of the first 20 amino acids of the mro reading frame, which are not found in the mature mutarotase, is similar to other signal sequences of exported proteins (29). A comparison of different procaryotic signal sequences with the first 20 amino acids of mutarotase is shown in Table III. The homology is not apparent at the level of the primary structure but rather at the level of charge and hydrophobicity distribution within the signal peptide (see Table III). All signal sequences shown in Table III

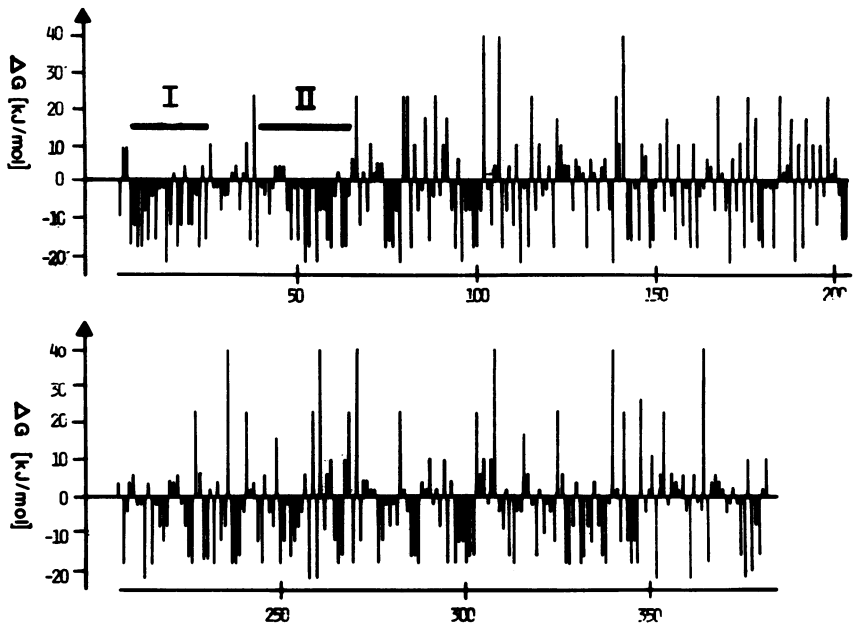

Fig. 3: Analysis of the free energy of transfer into the membrane for the mutarotase primary structure

The analysis was done as described (36). The figure shows the free energy of transfer of each amino acid from a random conformation in an aqueous environment into an α-helical structure in the membrane. The numbers refer to the position of the respective amino acid in the sequence of mutarotase. Two regions at the N-terminus are marked I and II because they show a high probability of incorporation into the membrane. This analysis indicates that mutarotase is a hydrophobic protein. The distribution of hydrophilic and charged amino acids is rather regular indicating that mutarotase is probably not located in the membrane.

contain one or two lysine residues following the methionine at their N-termini. Then a stretch of 14 to 19 hydrophobic amino acids is found. The amino acid preceeding the cleavage site is alanine with the only exception of serine for the fd coat protein (30). Judged by these criteria the first 20 amino acids in the mro reading frame constitute a leader peptide, which triggers secretion through the inner membrane of gram negative bacteria. The findings that mutarotase is located in the periplasmic space of A. calcoaceticus (W. Ebeling, pers. commun.), that it is excreted into the culture broth of E. coli WH1372 (see Figure 4) and that the cell associated portion in WH1372 can be released by osmotic shock treatment (see Figure 5), indicate that the leader peptide is indeed functional in both orga-

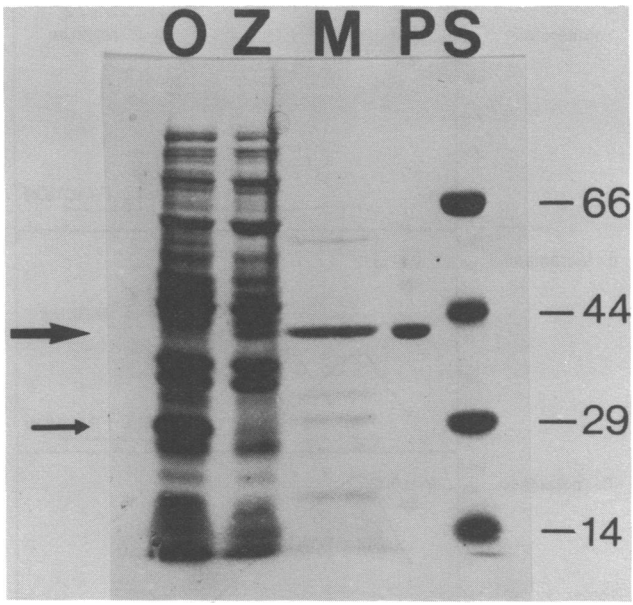

Fig. 4: SDS gel analysis of proteins associated with cells and in the culture medium of overproducing E. coli strains

The lane O shows the protein pattern of E. coli WH602 cells which overproduce the pSC101 encoded Tet repressor at nine hours after induction (16). The small arrow on the left indicates the position of this cytoplasmic protein. Lane Z shows the cell associated proteins of E. coli WH1372 under the same conditions. Lane M shows the proteins located in the culture broth of E. coli WH1372. The same amount of culture broth was analyzed in lanes Z and M. Lane P shows purified mutarotase from A. calcoaceticus. The position of the mature mutarotase is indicated by the heavy arrow on the left of the figure. Lane S shows molecular weight standards which are given in kDa on the right of the figure. A Western blot analysis using a mutarotase specific antibody revealed no signal in lane O and single bands precisely at the position of the large arrow in lanes Z, M, and P.

nisms. It has been shown before that the N-terminal amino acid sequences of mutarotase prepared from A. calcoaceticus and E. coli WH1372 are identical (45) It is therefore assumed that the signal sequence is specifically cleaved off by a leader peptidase at identical sites in both organisms.

An analysis of the primary structure of mutarotase regarding the free energy of transfer for each single amino acid from the aqueous phase to an α-helix inside the membrane is shown in Figure 3 (37). This graph indicates that two N-terminal sequences of 20 amino acids each have a high probability of forming α-helices inside the membrane. These sequences are denoted I and II in Figure 3. This feature of the primary structure is

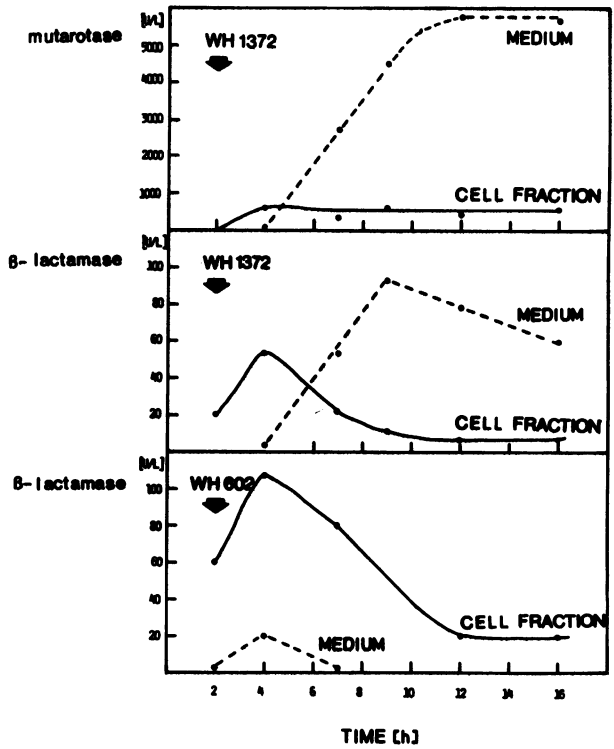

Fig. 5: Location of periplasmic proteins in E. coli WH1372 and WH602

The expression and location of mutarotase and ß-lactamase is shown for E. coli WH1372 and WH602. The upper curve gives the mutarotase activity associated with the cells (solid line) for E. coli WH1372 as a function of time after induction of expression (arrow). The dashed line indicates the activity found in the culture broth after spinning down the cells. The mutarotase activity associated with the cell fraction decreases to less than 50 U/l after osmotic shock treatment of the cells or after treatment of the culture with 0.2% trition X100 for 1 min at 42°C. The middle curve shows the ß-lactamase expression and location in the same experiment using the same symbols. The lower curve shows the expression and location of ß-lactamase in E. coli WH602 using the same symbols. E. coli cells were grown at 30°C in Luria broth (18) for 2h until the absorption at 650 nm had reached 0.6. Then the growth temperature was elevated to 42°C (arrow). The strains grew to an A_{650} of 1.8 which was reached after 3h. Then the cell density remained constant during continuation of the experiment.

often found for secreted proteins and is believed to support a loop model for the excretion process (37).

Secretion of mutarotase from E. coli WH1372 into the culture medium

Specific excretion of proteins into the culture medium is a very unusual event in E. coli. It has only been described so far for hemolysin (10) and

other endotoxins (11,12). Therefore, the excretion of mutarotase from E. coli WH1372 was characterized. Figure 4 shows an analysis of the cell associated proteins of E. coli WH1372 and E. coli WH602 at nine hours after induction. E. coli WH602 produces the same amount of a cytoplasmic protein, the pSC101 encoded Tet repressor, at the same time after induction from the same genetic background (16). It was used as a control overproducing a cytoplasmic protein. The protein patterns of both strains are nearly identical except for the Tet repressor band at 27 kDa in WH602 and the mutarotase band at 40 kDa in WH1372. The proteins in the culture broth were also analysed. The broth of E. coli WH1372 contains one major protein band at 40 kDa and several faint bands. The major protein component of this lane comigrates with purified mutarotase from A. calcoaceticus (lane P). The specific mutarotase activity in the culture broth is 60 U/mg protein and the one of purified mutarotase is 200 U/mg protein. The culture broth of E. coli WH602 contains only faint bands and is not shown in Figure 4. The clear difference in the protein patterns of the cell fraction and the culture medium indicates, that disruption of cells cannot be the cause for the occurence of mutarotase in the culture medium. As was already concluded from the leader sequence (see above) these data also support the notion that at least the secretion of mutarotase across the inner membrane has to be a specific process.

A Western blot analysis with a mutarotase specific antibody (38) gave no signal with the E. coli WH602 proteins while a single band at 40 kDa was labelled in the lane containing the E. coli WH1372 cell associated proteins and the same molecular weight was recognized by the antibody in the culture medium lane and in lane P (see Figure 4). It is interesting to note, that no precursor band was detectable in the Western blot of the E. coli WH1372 cell associated proteins.

In order to test for the specificity of excretion through the outer membrane the location of ß-lactamase was determined in dependence of mutarotase and Tet repressor expression. ß-lactamase has been well charcterized as a periplasmic protein (23). Figure 5 shows the expression and location of mutarotase and ß-lactamase in E. coli WH1372 and E. coli WH602, respectively, as a function of time after induction. Figure 5 demonstrates clearly, that the leakage of mutarotase from E. coli WH1372 into the culture medium is accompanied by leakage of the same percentage of ß-lactamase into the culture medium. The ß-lactamase produced in E. coli WH602 remains cell associated at all times after induction of Tet repressor synthesis. Compa-

rison of the ß-lactamase activity in E. coli WH1372 and E. coli WH602 indicates that the enzyme seems to be more stable in the culture medium than in the periplasmic space.

Taken together, the results presented in Figure 5 suggest that the accumulation of mutarotase from A. calcoaceticus in the periplasmic space of E. coli WH1372 destabilizes the outer membrane of that strain. This results in non-specific leakage of the periplasmic proteins mutarotase and ß-lactamase into the culture medium. It is asssumed that this leakage may affect all periplasmic proteins. Therefore, the excretion of mutarotase appears to be the result of specific secretion into the periplasmic space and non-specific leakage through the outer membrane.

DISCUSSION

In this article we describe for the first time the nucleotide sequence of a gene from A. calcoaceticus. Although this organism is not related to E. coli, the codon usage of the mro gene analyzed here is for most amino acids similar to the ones of weakly expressed genes in E. coli (25). Major differences are only found for the codon usage of Leu, Ala, Gln and Arg (see Table II). Despite this result the expression of mutarotase in E. coli reaches 5% of the total proteins (45) indicating that this odd codon usage does not lead to severe interference with translation in E. coli.

The nucleotide sequence preceeding the ATG start codon shows good homology to the translation initiation sites in E. coli (39,40). Instead of AGG six to nine nucleotides 5' of ATG the mro gene has AGT six and AGA eight nucleotides preceeding the ATG. Futhermore, three nucleotides 5' of ATG is no G and the positions 5 to 10 3' of A from the ATG are rich in A and T. These homologies to the genes of E. coli indicate that Acinetobacter uses similar signals for the initiation of translation. This lends additional support to the assumption that the ATG in position 121 (see Figure 2) is indeed the start codon for the mutarotase precursor.

The initiation site of transcription is not obvious from homologies with E. coli promotors (41). No termination of another open reading frame was found within 200 bp upstream of the mro gene (data not completely shown). Therefore, this nucleotide sequence could contain the promotor for the mro gene. A search for homologies to the E. coli promotor consensus sequence, however, did not lead to a suggested promotor. Since promotor sequences from Acinetobacter are not available to date, the identification of the mro transcription start site will have to be done by a functional assay.

The nucleotide sequence of the mro gene reveals the excistance of a leader peptide with clear homology to other signal sequences (see Table III). This implies that mutarotase is synthezised as a precursor and that 20 amino acids are cleaved upon secretion across the inner membrane. This signal is active in both E. coli and A. calcoaceticus and the proteolytic cleavage site is identical in both organisms (45). An attempt to identify the precursor in a Western blot analysis failed. This is in agreement with the notion of cotranslational processing (37). The analysis of the primary structure of mutarotase for the free energy of incorporating each amino acid in an α-helix inside the membrane reveals two regions with a large negative ΔG in the N-terminal part of the protein (see Figure 3). This finding supports the loop model for secretion of the mutarotase through the inner membrane (37). The sequence of the leader peptide, the location of mutarotase in the periplasmic space of A. calcoaceticus (W. Ebeling, personal communication), the SDS gel analysis in Figure 4, and the result that the cell accociated fraction of mutarotase in E. coli WH1372 is released by osmotic shock treatment support the conclusion that secretion of the enzyme across the inner membrane is a specific process.

In contrast, the secretion across the outer membrane appears to be a nonspecific process resulting from the high concentration of mutarotase in the periplasmic space. This conclusion is strongly suggested by the identical time courses of leakage of mutarotase and ß-lactamase from E. coli WH1372 (see Figure 5). A similar result has been observed before for the expression of penicillinase in E. coli (13). However, for technical production of mutarotase on a large scale secretion of the enzyme into the culture broth is of advantage because it is an enrichment of the product and all cell associated proteins can be removed by centrifugation.

Acinetobacter is a versatile organism with the ability ot adopt to a great variety of environments and to use a large spectrum of carbon sources (42). Furthermore, plasmids have been identified in that organism (43) and highly competent strains have been described (44). Therefore, the cloning and sequence analysis of the first gene from Acinetobacter may be helpful to develop a cloning system and study gene expression and their regulatory mechanisms in this interesting organism.

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