A direct sulfhydrylation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa*

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The relationship between genes and enzymes in the methionine biosynthetic pathway has been studied in *Pseudomonas aeruginosa*. The first step is catalysed by an O-succinylhomoserine synthase, the product of the *metA* gene mapped at 20 min on the chromosome. The second step is achieved by direct sulfhydrylation, involving the enzyme encoded by a *metZ* gene that we have identified and sequenced, located at 40 min. Thus *Pseudomonas* appears to be the only organism so far described that uses O-succinylhomoserine as substrate for a direct sulfhydrylation. As in yeast, the two transsulfuration pathways between cysteine and homocysteine, with cystathionine as an intermediate, probably exist in parallel in this organism.

Keywords: methionine biosynthesis, *Pseudomonas*, sulfhydrylation

INTRODUCTION

The biosynthetic pathway leading to methionine has been studied in many organisms and presents several characteristics that are interesting from an evolutionary point of view, as differences are apparent when different organisms are compared (see Fig. 1).

The first step, acylation of homoserine, is common to all organisms, but differs in the acyl group transferred. In enteric bacteria, the reaction is a succinylation (catalysed by a homoserine O-succinyltransferase, the product of the *metA* gene in *Escherichia coli*; Michaeli & Ron, 1981); it has been shown to be an acetylation by acetyl-CoA in *Saccharomyces cerevisiae* (homoserine O-acetyltransferase; the MET2 gene product; Langin *et al.*, 1986) and in *Brevibacterium flavum* (Ozaki & Shii, 1982), whereas in plants O-phosphorylhomoserine has been identified as the natural form of activated homoserine (Macnitol *et al.*, 1981). The last step is also similar among different species, a methionine synthase activity converting homocysteine to methionine (catalysed in *E. coli* by the products of the *metE* and *metH* genes; Chu *et al.*, 1985; Greene *et al.*, 1973).

The major differences depend upon the way the assimilation of the sulfur atom into the amino acid backbone is achieved and the way the so-called transsulfuration pathway occurs (for review, see Yamagata, 1989). Sulfur can be incorporated into a three-carbon derivative (a serine ester) yielding cysteine, or into a four-carbon derivative (a homoserine ester) yielding homocysteine. Homocysteine and cysteine can then be converted into one another by transsulfuration, which transfers the sulfur atom with the intermediary formation of cystathionine. Synthesis of cysteine from homocysteine (the C4 to C3 pathway) is the only pathway that exists in mammals. In enteric bacteria, the C3 to C4 pathway operates from cysteine and the homoserine ester; cystathionine γ -synthase (the *E. coli metB* gene product; Duchange *et al.*, 1983) and cystathionine β -lyase (the *E. coli metC* gene product; Belfaiza *et al.*, 1986) are successively involved (Fig. 1a). Yeast (Cherest & Surdin-Kerjan, 1992) and *B. flavum* (Ozaki & Shii, 1982) are capable of incorporating reduced sulfur into the homoserine ester by direct sulfhydrylation, a reaction catalysed by an acetyl-homoserine sulfhydrylase (the MET25 gene product in *S. cerevisiae*; Kerjan *et al.*, 1986); homocysteine is then the precursor of both methionine and cysteine, C4 to C3 transsulfuration being the only pathway used to synthesize cysteine in yeast. Moreover, in *S. cerevisiae* (Fig. 1b) it has been shown that the reverse transsulfuration pathway exists in parallel, since a MET25 mutant can grow on cysteine as a source of methionine (Kerjan *et al.*, 1986).

In *Pseudomonas aeruginosa*, somewhat contradictory results are reported in the literature. Calhoun & Feary (1969)

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Abbreviation: PLP, pyridoxal 5-phosphate.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence data reported in this paper is U10904.

Fig. 1. The methionine biosynthetic pathway in (a) *E. coli* and (b) *S. cerevisiae*.

have isolated and characterized methionine and cysteine auxotrophs. The growth requirements of these mutants suggested to the authors that the route used was similar to the one described for *E. coli*. Gunther *et al.* (1979) and Schelenz *et al.* (1986) isolated mutants in the different steps of sulfate assimilation; the growth requirement of these mutants could be satisfied by methionine alone, whereas no strict cysteine auxotroph could be isolated. This result suggests the possibility of interconversion of these two amino acids.

In order to obtain a better understanding of the situation, we have undertaken a more precise identification of two mutations mapped on the *P. aeruginosa* chromosome (*met-28* and *met-9011*, located at 20 and 40 min, respectively; O'Hoy & Krishnapillai, 1987) and have obtained evidence for the involvement of an *O*-succinylhomoserine sulphydrylase in methionine biosynthesis in *P. aeruginosa*.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Bacteria were grown as described previously (Martin *et al.*, 1986). Sulfate-free medium was an M9 medium (Maniatis *et al.*, 1982),

except that $MgCl_2$ was used in place of $MgSO_4$. Inorganic sulfur sources were added as described by Cherest & Surdin-Kerjan (1992). IPTG was used at 1 mM or 5 mM for *E. coli* or *P. aeruginosa* cultures, respectively.

The conjugative properties of pRK2013 (Figurski & Helinski, 1979) were used to transfer recombinant plasmids from *E. coli* to *P. aeruginosa*. *Pseudomonas* transconjugants were then screened on *Pseudomonas* isolation agar (PIA; Difco) supplemented with antibiotics at the following concentrations: for *E. coli* ampicillin (Ap), 50 $\mu g\ ml^{-1}$ and streptomycin (Sm), 100 $\mu g\ ml^{-1}$; for *P. aeruginosa* carbenicillin (Cb), 300 $\mu g\ ml^{-1}$ and streptomycin (Sm) 1 $mg\ ml^{-1}$.

DNA methods. DNA fragments used in subcloning were purified by low-melting-point agarose gel electrophoresis. Cleavage with restriction endonucleases, ligation and transformation were done as described in Maniatis *et al.* (1982). Sequencing on both strands was done as described by Bally *et al.* (1992). Various endonuclease restriction fragments encompassing the 1.9 kb *HindIII*-*SmaI* insert were obtained from the plasmid pMM18 (Filloux *et al.*, 1989; see Fig. 2).

Enzymic assays. Cell extracts were prepared by sonication. *O*-Acetylhomoserine sulphydrylase was assayed as described by Wiebers & Garner (1967). The following concentrations were used: sodium sulfide, 10 mM; pyridoxal 5-phosphate (PLP), 0.2 mM; *O*-succinylhomoserine (or *O*-acetylhomoserine), 20 mM; Tris, pH 8, 0.1 M; final volume 0.1 ml. The homocysteine formed was estimated according to the method described by Kredich & Tomkins (1966). Protein concentrations were estimated by the biuret method.

Chemicals. All reagents, including amino acids, were Sigma grade. *O*-Acetylhomoserine was a generous gift from Dr Y. Surdin-Kerjan.

RESULTS

Growth requirements of mutants

The growth requirements of strains PAO8 (*met-28*) and PAO503 (*met-9011*) were studied and compared to the wild-type PAO1. In both strains (see Table 2), methionine auxotrophy was confirmed, and homocysteine could replace methionine. Growth (slower than normal) was observed on cysteine with PAO503 but not with PAO8 (similar results were obtained on sulfate-free medium). None of the other compounds tested led to growth of the mutants. This can be easily explained in the case of *O*-succinylhomoserine and *O*-acetylhomoserine, as it is usually observed that these acylated compounds are poorly transported into bacteria. The permeation of cystathionine in *P. aeruginosa* is not known.

From these results, the following hypotheses can be made: (i) interconversion between cysteine and methionine is possible in strain PAO503, as already proposed by Gunther *et al.* (1979) and Schelenz *et al.* (1986); (ii) strain PAO503 should be blocked in the last steps prior to cysteine biosynthesis; (iii) the *met-28* mutation must affect one gene corresponding to *metA*, *metB* or *metC*.

Complementation tests

Heterologous complementation was tested using the *E. coli metA* and *metB* genes. These genes were cloned downstream of a *tac* promoter, which is known to be

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
<i>P. aeruginosa</i>		
PAO1	Prototroph (<i>chl-2</i>)	B. W. Holloway collection
PAO8	<i>met-28 ilv-202 str-1</i>	B. W. Holloway collection
PAO503	<i>met-9011</i>	B. Wretling
<i>E. coli</i>		
MC4100	<i>araD ΔlacU169 rpsL relA thi</i>	Maniatis <i>et al.</i> (1982)
β180	<i>metA::Cm^R</i>	Richaud <i>et al.</i> (1993)
WA802	<i>metB1 lac galK2 galT22 supE44 hsdR2</i>	Richaud <i>et al.</i> (1993)
Plasmids		
pRK2013	ColE1, Tra ⁺ Mob ⁺ , Km ^R	Figurski & Helinski (1979)
pMMB67HE	whr RSF1010 replicon, <i>tac</i> promoter, Ap ^R /Cb ^R	Fürste <i>et al.</i> (1986)
pUC9 and pUC13	ColE1, <i>lacI'</i> , Φ80 <i>dlacZ</i> , Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pMA61	<i>E. coli metA</i> cloned in pUC13	Richaud <i>et al.</i> (1993)
pMB41	<i>E. coli metB</i> cloned in pUC9	Richaud <i>et al.</i> (1993)
pMMA1	2.1 kb <i>EcoRI</i> – <i>HindIII</i> fragment from pMA61 containing <i>E. coli metA</i> cloned in pMMB67HE	This work
pMMB17	1.3 kb <i>EcoRI</i> – <i>HindIII</i> fragment from pMB41 containing <i>E. coli metB</i> cloned in pMMB67HE	This work
pGSS8	IncQ, Tc ^R , Sm ^R	Sharpe (1984)
pMM18	pGSS8 containing a 10 kb PAO1 DNA fragment	Filloux <i>et al.</i> (1989)
pMP5	1.9 kb <i>HindIII</i> – <i>SmaI</i> fragment from pMM18 cloned in pMMB67HE	This work

PAO1 chromosome

Fig. 2. Genetic organization and restriction endonuclease map of the *metZ* region. The most probable order of the genetic markers in the 40 min region is based on the genetic map of *P. aeruginosa* (Holloway & Zhang, 1990) and on previous data (Akrim *et al.*, 1993). The DNA fragment originated from pMM18 (Filloux *et al.*, 1989). The 1.9 kb sequenced fragment containing *metZ* is shown in filled bars. Restriction sites are indicated as follows: E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SalI*; Sm, *SmaI*.

operative in *Pseudomonas* (Fürste *et al.*, 1986), on plasmid pMMB67HE (see Table 1) leading to pMMA1 and pMMB17, respectively; these hybrid plasmids were transferred by triparental mating in the *Pseudomonas* mutant strains. Results are given in Table 3.

The defect of strain PAO8 is complemented by expression of the *E. coli metA* gene. This indicates that the *met-28* mutation affects the corresponding *Pseudomonas* gene and that O-succinylhomoserine should be the normal methionine precursor in *Pseudomonas* as in *E. coli*.

The defect in strain PAO503 is complemented by the expression of the *E. coli metB* gene. As this enzyme normally uses cysteine and O-acylhomoserine for cystathionine synthesis, this result should indicate that cysteine must be synthesized in the mutant (contradicting our preceding hypothesis). On the other hand, the possibility that the *met-9011* mutation could affect a cystathionine-synthase-encoding gene is in contradiction with the fact that the mutant can grow in the presence of cysteine alone. As the corresponding wild-type DNA fragment had been isolated by Filloux *et al.* (1989), a 1.9 kb *HindIII*–*SmaI* DNA fragment containing the gene was subcloned on pMMB67HE downstream of a *tac* promoter leading to plasmid pMP5. When pMP5 was introduced into PAO503

Table 2. Growth requirements

Inocula were prepared from bacteria grown on minimal medium plus methionine. Growth was estimated after 24 h. ++, Normal growth; +, growth slower than normal; -/+ , poor growth; -, lack of detectable growth. Abbreviations: OSH, O-succinylhomoserine; OAH, O-acetylhomoserine; Cyst, cystathionine; Hcys, homocysteine.

Supplement		Strain		
		PAO1	PAO503	PAO8
Minimal medium	None	++	-	-
	OSH or OAH	++	-	-
	Cyst	++	-	-
	Hcys	++	++	++
	Cys	++	+	-
	Met	++	++	++
Minimal SF*	None	-/+	-	-
	Sulfate, sulfite or sulfide	++	-	-
	Cyst	++	-	-
	Hcys	++	++	++
	Cys	++	+	-
	Met	++	++	++

* Sulfate-free medium.

and WA802 (*E. coli metB*), complementation was obtained with the former but not with the *E. coli* mutant strain (negative results were also obtained with other *E. coli* methionine auxotrophs; not shown). This negative result led us to think that the *E. coli* pathway is not operative in *Pseudomonas* and that this species follows the yeast pathway, using O-acylsulfhydrylase activity.

Measurements of such an activity were thus performed in the different strains. Results are given in Table 4. Whereas

Table 4. O-Succinylhomoserine sulfhydrylase activity

O-Succinylhomoserine (OSH) sulfhydrylase specific activities (nmol homocysteine produced min⁻¹ mg⁻¹) were determined on cell-free extracts as described in Methods. Bacteria were grown in minimal medium in the presence of IPTG. Plasmids are described in Table 1. Values are the means of at least three independent experiments. ND, Not detectable in our assay conditions.

Strain	Plasmid		OSH-sulfhydrylase specific activity
	Name	Gene cloned	
<i>P. aeruginosa</i>			
PAO1	None		12
PAO503 (<i>metZ</i>)	None		ND
	pMMB67HE	None	ND
	pMMB17	<i>metB</i>	ND
	pMP5	<i>metZ</i>	11.6
<i>E. coli</i>			
MC4100	pMMB67HE	None	ND
WA802 (<i>metB</i>)	pMMB67HE	None	ND
	pMMB17	<i>metB</i>	ND
	pMP5	<i>metZ</i>	11

no activity could be detected in *E. coli* as expected, O-succinylhomoserine is actively converted into homocysteine in *P. aeruginosa* wild-type cell-free extracts; it must be pointed out that O-acetylhomoserine was not a substrate, confirming the identity of the first step between *E. coli* and *Pseudomonas*. No activity could be detected in PAO503, whereas it was restored in this mutant harbouring the multicopy plasmid pMP5. The observed level is, in our experimental growth conditions in the presence of IPTG, similar to the one of PAO1 wild-type strain. This plasmid was also expressed in the *E. coli metB* mutant WA802

Table 3. Complementation tests

Bacterial growth was performed in the presence of IPTG and estimated as described in Table 2. Plasmids are described in Table 1. pMMB67HE is used as a control, pMMA1 carries the *E. coli metA* gene, pMMB17 carries the *E. coli metB* gene and pMP5 carries the *P. aeruginosa metZ* gene. Symbols are the same as in Table 2.

Strain	Genotype	Plasmid used for complementation:			
		pMMB67HE (vector)	pMMA1 (<i>metA</i>)	pMMB17 (<i>metB</i>)	pMP5 (<i>metZ</i>)
<i>E. coli</i>					
MC4100	Wild-type	+	+	+	+
β 180	<i>metA</i>	—	+	—	—
WA802	<i>metB</i>	—	—	+	—
<i>P. aeruginosa</i>					
PAO1	Wild-type	+	+	+	+
PAO8	<i>met-28</i>	—	+	—	—
PAO503	<i>met-9011</i>	—	—	+	+

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>PurF
1 CCTCCGCGAGCGTCCGACCACGACATCGACGTGGTTCATCCCGATCCCGGACACCAGCCGCACCGCGCGGCTGGAAGTGGCCAAACCGGCTCGGCGTGAAGT
P P R A S D H D I D V V I P I P D T S R T A R L E L A N R L G V K F
101 TCCGCGAGGGCTTCGTCAAGAACCGCTACATCGGCCGCACCTTCATCATGCCCGGCCAGGCGGCGCGGAAGAAATCCGTGCGGCAGAAGCTCAACGCCAT
R E G F V K N R Y I G R T F I M P G Q A A R K K S V R Q K L N A I
201 CGAGCTGGAGTTCGCGCGCAAGAACGTGATGCTGGTGGATGACTCCATCGTCCGCGGCACCCACCTGCAAGCAGATCATCCAGATGGCCCCGCGAGGCCGGG
E L E F R G K N V M L V D D S I V R G T T C K Q I I Q M A R E A G
301 GCGAAGAACGTCTACTTCTGCTCCGCCGCGCGGCCGTGCGCTACCCCAACGTCTACGGCATCGACATGCCGAGTGGCGACGAGCTGATCGCGCACAATC
A K N V Y F C S A A P A V R Y P N V Y G I D M P S A H E L I A H N R
401 GCAGCACCGAGGACGTGACGAAGCTGATCGGCGCGGACTGGCTGGTCTACCAGGACCTGCCGACCTGATCGACGCGGTGCGGCGCGGCAAGATCAAGAT
S T E D V S K L I G A D W L V Y Q D L P D L I D A V G G G K I K I
501 CGATCACTTCGACTGCGCGGTGTTCGACGGCGAGTACGTGACCGGCGACGTCAACGAGGCCTACCTGAACCGGATCGAACAGGCGCGCAACGATGCGACC
D H F D C A V F D G E Y V T G D V N E A Y L N R I E Q A R N D A T
601 AAGGCCAAGAGCCAGGCGGTGAGCGCATCATCGATCTGTACAACGACTGACAAAGGGGAGAGCGGCATGACTCAGGACTGGGATGCCGGCGGTCTCGAC
K A K S Q A V S A I I D L Y N D *
>MetZ
701 AGCGACCTGGAAGGCGCGGCCTTCGACACCTGGCGGTTTCGCGCGGGCAACGGCGGACGCGGAGGGCGAGCAGCGCGAGGCGGTGTTTACCACCTTCCA
S D L E G A A F D T L A V R A G Q R R T P E G E H G E A L F T T S S
801 GCTACGTCTTCGCGACCGCGCGCGGCGCGGTTTCGCGCGGAAGTGGCGGGCAACGTCTATTTCGCGCTACACCAACCCACGGTACGCACCTT
Y V F R T A A D A A A R F A G E V P G N V Y S R Y T N P T V R T F
901 CGAGGAGCGCATCGCCGCCCTCGAAGGAGCCGAGCAGGCGGTGGCCACGGCGTGGGCGATGTCGGCGATCCTCGCCCTGGTGTGAGCCTGTGCAGCTCC
E E R I A A L E G A E Q A V A T A S G M S A I L A L V M S L C S S
1001 GCGGACCACGTGCTGGTCTCGCGCAGCGTGTTCGGCTCGACCATCAGCCTGTTTCGACAAGTACTTCAAGCGCTTCGGTATCCAGGTGCGACTATCCGCCGT
G D H V L V S R S V F G S T I S L F D K Y F K R F G I Q V D Y P P L
1101 TGAGCGATCTCGCGGCCCTGGGAGGCGGCGTGAAGCCGAATACCAAGCTGTCTTTCGTGAGTCGCCATCCAACCCGCTGGCCGAGCTGGTGGATATCGC
S D L A A W E A A C K P N T K L F F V E S P S N P L A E L V D I A
1201 CGCGCTGGCCGAGATCGCCACGCCAAGGGTGCCTGCTGGCGGTGGACAAGTCTGTCACGCGGCGCTGCAACAGCCGCTGAAGCTCGGTGCGGAC
A L A E I A H A K G A L L A V D N C F C T P A L Q Q P L K L G A D
1301 GTGGTGATCCACTCCGCGACCAAGTACATCGACGCCAGGGCCGGGGCATGGGCGGGTGGTCCGCGTGGCGGAGCAGATGAAGGAAGTGGTGGCT
V V I H S A T K Y I D G Q G R G M G G V V A G R G E Q M K E V V G F
1401 TCCTCCGACCGCGCGGACCGACCCCTCAGCCCGTTCAACGCCTGGCTGTTCCTCAAGGCCCTGGAACCCCTGCGTATCCGCATGCAGGCGCACAGCGCCAG
L R T A G P T L S P F N A W L F L K G L E T L R I R M Q A H S A S
1501 TGCCCTGGCCCTGGCCGAATGGCTGGAGCGCCAGCGGGCATCGAGCGGTCTACTACGCGGCGCTGCCGAGCCATCCTCAGCATGAGTTGGCCCGCGC
A L A L A E W L E R Q P G I E R V Y Y A G L P S H P Q H E L A R R
1601 CAGCAGAGCGGCTTCGGCGCGGTGCTCAGCTTCGACGTGAAGGGCGGGCGCGATGCCGCCCTGGCGCTTCATCGACGCTACCCGGATGGTCTCGATACCA
Q Q S G F G A V V S F D V K G G R D A A W R F I D A T R M V S I T T
1701 CCAACCTGGGCGACACCAAGACCACCATCGCCACCCGGCGACACCTCCACGGTCGACTGTCCCGCAGGACCGCGCGCGCGCGGATCGGCGACAG
N L G D T K T T I A H P A T T S H G R L S P E D R A R A G I G D S
1801 CCTGATCCGGGTGCGGTCGGCCTGGAAGACCTCGACGACCTCAAGGCCGACATGGCTCGGGGGTGGCCGCGCTGTGATCGAACCCAACCTGCGAGGCA
L I R V A V G L E D L D D L K A D M A R G L A A L *
1901 ACGGCAAGGTGCGCCCTGGTCACCGGGGCGGCGCGG

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Fig. 3. Nucleotide sequence of the 1.9 kb fragment encoding MetZ.

(even though a negative complementation test for restoring growth was obtained).

These results led us to conclude that the DNA fragment cloned encodes a protein with *O*-succinylhomoserine sulphydrylase activity.

Sequence of the sulphydrylase gene

The nucleotide sequence of the 1.9 kb insert of pMP5 was determined on both strands as described in Methods. It is given in Fig. 3. A 1209 bp ORF (extending from position 668 to 1876) was evidenced. Its G + C content and a strong

Fig. 4. Sequence alignment of the *E. coli* MetB, the *S. cerevisiae* Met25 and the *P. aeruginosa* MetZ proteins. Boxed sequences of similarities are taken from Cherest *et al.* (1993). Asterisks indicate identical residues between the three sequences and dots indicate homologous residues. Residues shared by at least two proteins are in bold letters.

bias in the third position codon usage are typical of chromosomally encoded *Pseudomonas* genes (West & Iglewski, 1988). The corresponding protein (403 amino acids long; M_r 43186) is tentatively named MetZ.

Computer analysis indicated strong similarities with the *E. coli* MetB cystathionine γ -synthase and with the yeast *O*-acetylhomoserine sulfhydrylase (product of the *MET25* gene) (Fig. 4). These similarities (36.8% and 36.7% identity, respectively) are observed all along the sequences. The MetZ protein belongs to one of the two

families of transsulfuration enzymes recently defined by Cherest *et al.* (1993) including the rat and yeast cystathionine γ -lyases, the yeast homocysteine synthase, and both the cystathionine γ -synthase and cystathionine β -lyase from *E. coli*. For sake of clarity, Fig. 4 gives sequence alignments of only three sequences, and the conserved blocks observed by Cherest *et al.* (1993) are indicated; it can be seen that these blocks are also found in our sequence. Comparison with MetZ allows some additional observations: even though the calculated percentage of identical residues is globally similar when

comparing MetZ to Met25 or MetB entire sequences, *Pseudomonas* MetZ and yeast Met25 proteins display much longer conserved regions than *E. coli* MetB in their N-terminal part (till residue 90). The main difference between these two proteins is the additional presence of a stretch of about 30 amino acids in the yeast enzyme when compared to the two others. All these enzymes use PLP as a cofactor. In *E. coli* MetB Parsot *et al.* (1987) have shown that the Lys198 residue is covalently bound to PLP and Cherest *et al.* (1993) have already noted the strong conservation of this region; it is likely that Lys219 of MetZ must play a similar role.

The C-terminal part of an incomplete ORF (216 codons; position 1–648 in Fig. 3) was observed upstream of *metZ* which also possesses the characteristics of a *Pseudomonas* expressed sequence. Screening of the data banks indicated a very strong similarity (59.2% and 52.8% identity, respectively) with the C-terminal parts of the *E. coli* PurF (Tso *et al.*, 1982) and the *S. cerevisiae* Ade4 (Mäntsälä & Zalkin, 1984) glutamine phosphoribosylpyrophosphate amidotransferases. We thus propose this ORF to be called PurF; this is in agreement with the fact that a mutation leading to purine auxotrophy (*pur-9013*) has been localized in this region on the *Pseudomonas* chromosome (O'Hoy & Krishnapillai, 1987). The last codon of PurF is only 16 bp upstream from the ATG of MetZ.

DISCUSSION

In *P. aeruginosa* three loci have been described with mutations leading to a Met[−] phenotype: one is located at 20 min on the chromosome; a second at 40 min; the third at 31 min. In fact it has been shown by Clepet *et al.* (1992) that this last locus encodes homoserine dehydrogenase, the enzyme that synthesizes homoserine, the precursor of both threonine and methionine (growth of mutants in this gene is obtained in the presence of homoserine alone or threonine plus methionine).

We have studied mutations mapping at the two other loci by different approaches: determination of the growth requirements of the corresponding mutants, possibility of heterologous complementation, measurements of sulphhydrylase enzymic activity, and the sequencing of one locus.

At 20 min several Met[−] mutants were mapped (*met-28*; *met-60*; *met-66*; *met-8003*). They appear to be all linked, with the possible exception of *met-8003*, but we could not obtain this mutant from other laboratories. The *met-28* mutation was chosen as a representative of this class. It is complemented by the expression of the *E. coli metA* gene, in agreement with growth in the presence of homocysteine. Thus this region encodes an homoserine O-succinyltransferase activity, the product of the *Pseudomonas metA* gene. The fact that succinyl homoserine is the true acyl intermediate is strengthened by our data indicating that this ester is the only substrate used in the following enzymic step.

It is not known if other methionine genes are located in this region. However as the gene organization in the two

other loci is known (see below and Clepet *et al.*, 1992), and if we take account for the fact that mutants affecting all possible steps have been already isolated, one may postulate that the gene(s) encoding methionine synthase, the last step in the pathway, will also map in this 20 min region.

Our results show that the *met-9011* mutation (located at 40 min) affects a gene encoding O-succinylhomoserine sulphhydrylase activity; we propose to call this gene *metZ*. This is indicated by: (i) the loss of such an activity in the mutant strain, whereas it exists in the wild-type parental strain; (ii) the fact that a 1.9 kb DNA fragment carrying this region complements this mutation and expresses the sulphhydrylase activity both in *Pseudomonas* and in *E. coli*. Moreover conserved regions are observed in the N-terminal part of MetZ and the corresponding yeast enzyme that could reflect functional similarities.

The sequence of the fragment was entirely determined and no other entire ORF was present. Moreover the sequence of 14 contiguous kb is actually known in this region, and no genes were found to be homologous to any methionine genes: downstream is an *orfX*, encoding a putative protein similar to FixR of *B. japonicum* (Akrim *et al.*, 1993), followed by several *xcp* loci involved in protein secretion (Bally *et al.*, 1992); upstream is a putative PurF-encoding sequence (this paper; see Fig. 2). Thus no other gene involved in methionine biosynthesis is located in this region. This eliminates the possibility that the *met-9011* mutation will have a polar effect, leading to pleiotropic requirements for intermediates of methionine biosynthesis and to difficulties in the interpretation of complementation tests.

Thus *Pseudomonas* differs from enteric bacteria by the existence of a direct sulphhydrylation pathway, as in *B. flavum* and *S. cerevisiae*, except that O-succinylhomoserine is used as the substrate instead of O-acetylhomoserine, which is used by the other two species. To our knowledge, *P. aeruginosa* is the only organism so far described using O-succinylhomoserine as substrate for direct sulphhydrylation.

Another important point is indicated by the fact that the mutant impaired in O-succinylhomoserine sulphhydrylase activity (but not the mutant lacking O-acylhomoserine synthesis) can fulfil its auxotrophy by cysteine. Though the direct sulphhydrylation has to be the major route (as indicated by the slow growth of the mutant on cysteine), the C3 to C4 transsulfuration pathway must exist in parallel. This could explain the apparently conflicting result we obtained by complementing the mutation affecting the sulphhydrylase activity by overexpression of the *E. coli metB* gene; such an increase in cystathionine γ -synthase activity would make the transsulfuration pathway more effective for synthesizing homocysteine from cysteine; in this case cysteine would be synthesized from serine in *Pseudomonas* as in *E. coli*. However, due to the overall similarity of the phenotypes we favour the hypothesis that *Pseudomonas* follows the global pathway established for *S. cerevisiae* by Cherest & Surdin-Kerjan (1992), in which serine sulphhydrylase is inoperative and

cysteine is synthesized only by the reverse C4 to C3 transsulfuration pathway. In such a situation, complementation of the *Pseudomonas metZ* mutant by the *E. coli metB* gene could be explained as proposed by Richaud *et al.* (1993). These authors have recently observed that *metB* overexpression in *E. coli* allows homocysteine synthesis in a strain with a *metC* disrupted gene. They suggest that a bypass of cystathionine through the condensation of a sulfur moiety other than cysteine with *O*-succinyl-homoserine could be challenged by the MetB enzyme, as already suggested by the work of Flavin & Slaughter (1967), who observed the existence of a 'parasite' sulfhydrylase activity *in vitro* for this protein, and of Simon & Hong (1983), who isolated *E. coli metQ* mutants displaying a phenotype similar to the one obtained by Richaud *et al.* (1993). As the *Pseudomonas MetZ* protein is inoperative for complementing *E. coli metB* (and *metC*) mutants, we have to suppose that the sulfur donor is not the same, or has quite different affinities, for the MetZ and MetB proteins. Isolation of mutants similar to those isolated in *S. cerevisiae* would be necessary to prove that *Pseudomonas* strictly follows the yeast pathway as described in Fig. 1(b).

One might note that such a relationship between yeast, *Brevibacterium* and *Pseudomonas* has already been noted when comparing identical enzymes from different organisms: a stronger similarity was observed between *Pseudomonas* and yeast proteins and *Pseudomonas* and *Brevibacterium* proteins than between *Pseudomonas* and *E. coli* (Cami *et al.*, 1993).

Finally, one may wonder why cystathionine cannot replace cysteine for the growth of strains PAO503. Two phenomena, acting together, may account for this. First, the uptake of cystathionine could be poorly achieved in *Pseudomonas*. Cystathionine auxotrophs have been described in *Pseudomonas* (Calhoun & Feary, 1969); however, the authors indicated that growth was very poor in the presence of cystathionine and that the chemical product used in their tests was not chromatographically pure. Such a poor permeation in this species compared to *E. coli* has already been proposed in the case of diaminopimelate (Martin *et al.*, 1986). Second, the reaction synthesizing homocysteine, then methionine, from cystathionine could be poorly active in *Pseudomonas*, as it seems to be the case in yeast. In both such conditions, cystathionine would not be at a concentration high enough in the cell to allow both methionine and cysteine syntheses. These hypotheses would explain the results we obtained when growing cells in sulfate-free medium (Table 2). In such a medium, a residual sulfate concentration, as revealed by poor growth without addition, would allow methionine synthesis, with a sparing effect of cystathionine used as a precursor of cysteine; this would not be possible in the mutant strain PAO503. As similar phenotypes are observed in yeast (Pigg *et al.*, 1962; H. Cherest, personal communication), this reinforces our hypothesis that *Pseudomonas* and yeast follow the same global pathway.

The simultaneous presence of the two transsulfuration pathways in *Pseudomonas* raises the question of their physiological meaning. In this respect, it would be

interesting to know if differential expression of these pathways could be achieved according to regulatory mechanisms depending on specific growth conditions.

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