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Characterization of the *aes* Gene of *Escherichia coli* Encoding an Enzyme with Esterase Activity

RALF PEIST, ANDREAS KOCH, PETRA BOLEK, SVEN SEWITZ, THOMAS KOLBUS, AND WINFRIED BOOS*

Department of Biology, University of Konstanz, D-78457 Konstanz, Germany

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malQ **mutants of *Escherichia coli* lacking amylomaltase cannot grow on maltose. They express the maltose system constitutively and are sensitive to maltose when grown on another carbon source. In an attempt to isolate a multicopy suppressor that would result in growth on maltose, we transformed a *malQ* mutant with a gene bank of *E. coli* DNA which had been digested with *Sau*3a and cloned in pBR322. We screened the transformants on MacConkey maltose plates. A colony was isolated that appeared to be resistant to maltose and was pink on these plates, but it was still unable to grow on minimal medium with maltose as the carbon source. The plasmid was isolated, and the gene causing this phenotype was characterized. The deduced amino acid sequence of the encoded protein shows homology to that of lipases and esterases. We termed the gene *aes*, for acetyl esterase. Extracts of cells harboring plasmid-encoded *aes* under its own promoter exhibit a fivefold higher capacity to hydrolyze *p*-nitrophenyl acetate than do extracts of cells of plasmid-free strains. Similarly, strains harboring plasmid-encoded *aes* are able to grow on triacetyl glycerol (triacetin) whereas the plasmid-free strains are not. The expression of plasmid-encoded *aes* resulted in strong repression of the maltose transport genes in *malT*$^+$ strains (10-fold reduction), but not in a *malT*(Con) strain which is independent of the inducer. Also, overproduction of MalT counteracted the Aes-dependent repression, indicating a direct interaction between MalT and Aes.**

The *Escherichia coli* maltose system consists of a number of genes encoding enzymes and envelope-associated proteins involved in the specific uptake and degradation of maltose and maltodextrins (8, 42). The regulation of this system is based on the function of MalT, which becomes an activator in the presence of cyclic AMP-catabolite activator protein, ATP and maltotriose, the specific inducer of the system (38). One of the many peculiarities of the maltose system is the phenomenon of endogenous induction that is caused by the internal production of maltotriose. Thus, even in the absence of maltose or maltodextrins in the growth medium, the system is turned on at a low level due to endogenously produced maltotriose (16). Aside from glycogen, which is a possible source of maltotriose, we have demonstrated that maltose and maltotriose can be produced from internal glucose and glucose-1-phosphate and have postulated that this is due to an enzymatic activity resembling a maltose/maltotriose phosphorylase (16). Even though the enzymatic activity can easily be seen in intact cells, we were unable to find mutations in the gene coding for this enzyme (31). In an attempt to clone the gene encoding the postulated maltose/maltotriose phosphorylase, we argued that this enzyme should catalyze its reaction reversibly and should also form glucose and glucose-1-phosphate from maltose with inorganic phosphate. Therefore, when present in large amounts, this enzyme should lead to growth on maltose.

malQ mutants lacking the activity of amylomaltase, the key enzyme in the degradation of maltodextrins, are unable to grow on maltose but are sensitive to it since they accumulate this sugar to high internal concentrations. We used a *malQ*::Tn*10* mutant as the recipient for an *E. coli* gene bank and MacConkey maltose indicator plates as a screen for growth. On these plates, the starting strain forms pale, flat colonies that quickly develop papillae and that outgrow the original strain as normal-sized pale colonies consisting mainly of *malK* (transport) and *malT* (regulation) mutants (42). After transformation with the gene bank, all Mal$^+$ strains found in the selection contained a plasmid harboring the *malQ*$^+$ gene. However, we found one mutant exhibiting a pink but maltose-resistant phenotype that was due to a plasmid-encoded gene. Even though

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
BRE1161	MC4100Φ(*malT-lacZ*)(λp*lac*Mu50)	11
BRE1162	MC4100Φ(*malK-lacZ*)(λp*lac*Mu50)	11
BRE1163	MC4100Φ(*malE-lacZ*)(λp*lac*Mu50)	11
CB39	MC4100 *malQ*::Tn*10*	10
DS410T	*minB ara lacY malA mtl xyl rpsL thi tonA azi* Δ(*glpT-glpA*)*593*	26
JB3018	MC4100 *malT*(Con)	*malT*(Con) allele (44)
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 flbB5301 deoC1 relA1 rbsR rpsL150 ptsF25 thi-1*	14
RP104	MC4100 *aes*::Tn*10*(Cam^r)	This study
RP105	MC4100 *aes*::*lacZ* Kan^r	This study
RP110	BRE1161 *aes*::Tn*10*(Cam^r)	This study
RP111	BRE1162 *aes*::Tn*10*(Cam^r)	This study
RP112	BRE1163 *aes*::Tn*10*(Cam^r)	This study
Plasmids		
pAS1	pQE31 *aes* (under IPTG-inducible promoter), Ap^r	pQE31 from Qiagen
pBR322	Ap^r, Tc^r	5
pRP10	pBR322 Ap^r *aes*$^+$ *hemH*$^+$	This study
pRP11	pBR322 Ap^r *aes*$^+$	This study
pRP12	pRP11 Ap^r *aes*$^+$::Cam^r	This study
pRP13	pRP11 Ap^r *aes-lacZ*	This study
pRP31	pHSG575 *malT*$^+$ Cam^r	pHSG575 (46)

* Corresponding author.

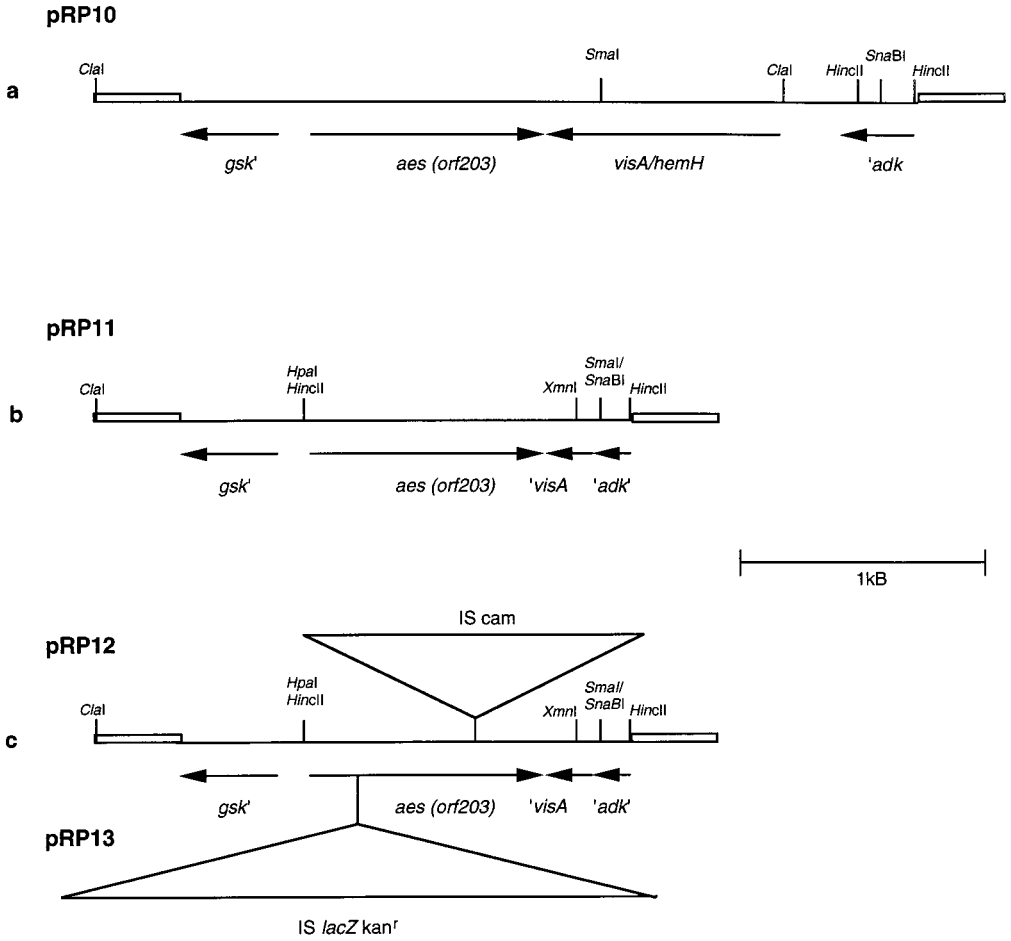

FIG. 1. Chromosomal position of *aes* and analysis of the plasmids used in this study. The position of *aes* is between *gsk* and *hemH*/*visA* at 10.8 min on the genetic map of *E. coli*. Plasmid pRP11 (b) was obtained from pRP10 (a) by deleting the *Sma*I-*SnaB*I fragment. The positions of the *aes*::cam and *aes*::*lacZ* insertions (IS) in pRP12 and pRP13 (c) are indicated. The open bars represent vector pBR322 into which the gene bank of partially digested *Sau*3a fragments was ligated at its *Bam*HI site.

this gene did not confer maltose growth on minimal maltose plates, it had altered the maltose sensitivity of the original *malQ* mutation, indicating utilization of maltose.

In this paper, we characterize the responsible gene, which we named *aes*. We found that the plasmid-encoded protein is a cytoplasmic enzyme exhibiting esterase activity. It counteracts maltose sensitivity by interfering with the expression of the maltose system.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic techniques. Derivatives of *E. coli* K-12 strains were used throughout this study. They were constructed by P1 vir transduction (27) and are listed in Table 1. Strains were grown at 37°C in Luria-Bertani (LB) broth, tryptone broth, NZA medium (10 g of NZ-amine A [Sheffield Products, Inc.) per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter), or minimal medium A (MMA) (27) supplemented with 0.2% carbon source. The antibiotics ampicillin, tetracycline, kanamycin, and chloramphenicol were used at final concentrations of 100, 5, 100, and 30 μg/ml, respectively. Chromosomal DNA was isolated as described previously (45). Standard recombinant DNA techniques were used (2, 40).

To screen for plasmid DNA coding for the postulated maltose/maltotriose phosphorylase, strain CB39 was electrotransformed with a gene bank of partially digested DNA of ECL116 cloned in the *Bam*HI site of pBR322 (15). The transformants were replica-plated on MacConkey plates containing 1% maltose and screened for red colonies.

Construction of plasmids. pRP11 was obtained by deleting an 1,186-bp *Sma*I-*Sna*BI fragment from pRP10. The in vivo construction of pRP12 harboring a Cam^r insertion in *aes* was done as described previously (22). Thus, strain MC4100(pRP11) grown overnight in MMA-maltose was infected with phage λNK1324 harboring the chloramphenicol resistance cassette Tn*10* (Cam^r) and selected for resistance to chloramphenicol. Plasmid DNA was isolated from pooled Cam^r clones, retransformed into CB39, selected for Cam^r, and screened for the loss of the ability to form pink colonies on MacConkey indicator plates containing 1% maltose. The plasmid DNA of one clone (pRP12) was sequenced to determine the position of the Tn*10* (Cam^r) insertion.

pRP13 coding for the *aes-lacZ* fusion was obtained by the same technique. λNK1205, a phage harboring a promoterless *lacZ* gene and a kanamycin resistance cassette, was used. In this case, the necessary transposase was encoded on a second plasmid (pNK2882).

pAS1 carrying *aes* under an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter was constructed in the following way. The 960-bp fragment containing the complete open reading frame of *aes* was amplified by PCR from pRP11 with two flanking primers containing a *Bam*HI cleavage site at the 5′ end and a *Sal*I cleavage site at the 3′ end. The resulting 985-bp fragment was cleaved with *Bam*HI and *Sal*I and ligated into pGDR11 downstream of an IPTG-inducible *lac* promoter by using the same restriction sites. pGDR11 is a derivative of pQE31 (from Qiagen) into which the *lacI*^{q} gene was ligated. The latter was amplified by PCR from pMal-P2 (from New England Biolabs) and cloned in the *Xba*I site of pQE31.

pRP31 is a derivative of pHSG575 (46) harboring *malT* under its own promoter. The 3,515-bp *Eco*RI-*Pst*I fragment holding the coding sequence of *malT* from pOm2 (33) was cloned into the *Eco*RI-*Pst*I-digested pHSG575 vector.

Determination of the transcriptional start point. The mRNA start point was mapped by the reverse transcriptase method of primer extension as described previously (1) with 20 to 50 μg of total cellular RNA and 5 pmol of the digoxigenin-labeled primer starting at bp 296 (see Fig. 2): 5′GGCTGAAGAGTATT CACAACGG3′. The sequencing reaction was done with the same primer.

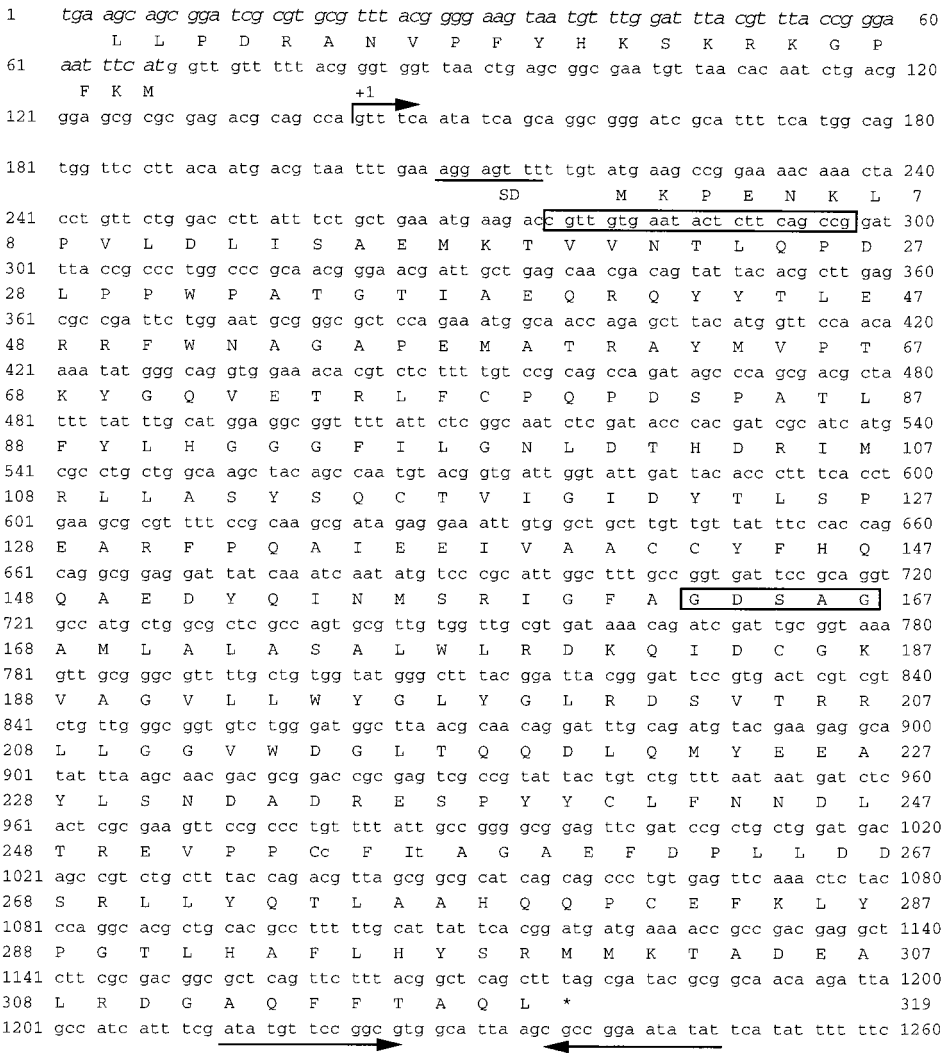

FIG. 2. Sequence of *aes* and the deduced amino acid sequence of Aes. The coding sequence starts with ATG at base 220 and ends at the stop codon TAG at base 1177. The arrow at base 142 points to the transcriptional start, and the two inverted arrows at the end of the sequence point to a potential transcriptional termination site. The boxed amino acid sequence represents the consensus sequence found in lipolytic enzymes (4). The boxed DNA sequence shows the primer used for determining transcription initiation. The DNA sequence in italics above the amino acid sequence (at the beginning of the DNA sequence shown) represents the divergently oriented *gsk* gene encoding guanosine kinase (19). *aes* is located at 10.75 min on the genetic map of the *E. coli* chromosome between *gsk* and *adk* and is transcribed in a counterclockwise orientation.

Exchange of wild-type *aes* on the chromosome for the Cam^r insertion or the *aes*::*lacZ* fusion. For the transfer of the *aes229*::Tn*10* (Cam^r) of pRP12 or the *aes-lacZ65* fusion (Kan^r) of pRP13 into the chromosome, the *polA* strain HSK42 was used (39). The plasmids that had become integrated in the chromosome were transferred by P1-mediated transduction into MC4100. A P1 lysate was then grown on the pooled transductants. This lysate was used to select for chromosomally encoded Cam^r (originating from pRP12) or Kan^r (originating from pRP13) colonies that were ampicillin sensitive and had thus lost the plasmid. The two strains were named RP104 and RP105, respectively. The correct position of the insertion was verified by Southern analysis of the chromosomal DNA of the two strains.

DNA sequencing. To sequence *aes*, plasmids pRP12 and pRP13 were used. The plasmids were prepared with the Qiagen kit (Qiagen GmbH, Hilden, Germany). Sequencing was done by the chain termination method (41) with the digoxigenin Taq-DNA sequencing kit from USB/Amersham, using the two 5′-digoxigenin-labeled primers Cam3′ (5′-CGTAACGGCAAAAGCACC-3′) and Cam5′ (5′-CTGCCTCCAGAGCCTG-3′) to sequence pRP12. Sequencing was done from double-stranded DNA (48). The Cam^r primers for sequencing pRP12 had their start points at the ends of the Cam^r cassette positioned within the *aes* gene. The universal "−40" *lac* primer was used to sequence pRP13. The sequencing reaction was analyzed with the GATC1500 direct blotting electrophoresis system and colorimetric detection on nylon membranes (32).

Enzymatic assays. The β-galactosidase activity of *lacZ* fusion strains was tested in permeabilized cells by the method of Miller (27). Enzymatic activity is given in micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute at room temperature. Aes activity was assayed by the method of Higgins and Lapides (20) and Krish (23). Cell extract (100 μl; obtained by disruption in a French pressure cell) was mixed with 850 μl of prewarmed (30°C) phosphate buffer (75 mM potassium phosphate [pH 7.0], 10 mM $MgSO_4$), and the reaction was started by the addition of 50 μl of *p*-nitrophenyl acetate (pNPAc; 100 mM in ethanol). The formation of *p*-nitrophenol from pNPAc was monitored photometrically at 405 nm for 10 min, and units of enzymatic activity are given in micromoles of pNPAc hydrolyzed per minute at 30°C. A molar extinction coefficient of 9,940 M^{-1} was used for *p*-nitrophenol at pH 7.0.

Transport of [^{14}C]maltose. Cultures were grown logarithmically in MMA containing 0.4% glycerol as carbon source to an OD_{578} of 0.5. When indicated, 1 mM maltose was added 1.5–2 h prior to harvesting. The cells were washed three times with MMA and resuspended in the same medium to an optical density at 578 nm of 0.5 (glycerol-grown cultures) or 0.1 (maltose-induced cultures). To 3 ml of this suspension, [^{14}C]maltose was added at room temperature, resulting in a final concentration of 52 nM. Samples (0.5 ml) were withdrawn after different time intervals, filtered through membrane filters (pore size, 0.45 μm; Schleicher & Schüll, Dassel, Germany), and washed with 10 ml of MMA. Radioactivity was determined in a liquid scintillation counter (Beckman LS 1801). The uptake rate

was determined from the linear increase in the accumulated radioactivity and is given as picomoles of substrate taken up per 10^9 cells per minute.

Plasmid-directed Protein synthesis. Plasmid-directed protein synthesis in the minicell-producing strain DS410T (26) was performed by the method of Reeve (35). Labeling with 12 μCi of [^{35}S]methionine (1,000 mCi/mmol; Amersham Corp.) was carried out for 15 min at 37°C. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed with 12% gels by standard techniques (25). The samples were routinely heated for 3 min in boiling water.

Preparation of periplasmic proteins. Periplasmic proteins were prepared by the cold osmotic shock procedure of Neu and Heppel (29).

RESULTS

Isolation of the *aes*-carrying plasmid. The *malQ*::Tn*10* strain CB39 was transformed with a gene bank of partially *Sau*3a-digested DNA of strain ECL116 cloned into the *Bam*HI site of pBR322 (15). About 20,000 transformants were screened on MacConkey maltose plates in the presence of ampicillin. Seven transformants exhibited a strong Mal$^+$ phenotype. According to the restriction analysis of their plasmid DNAs, they all contained the *malQ*$^+$ gene and were not considered further. One transformant exhibited a pink phenotype and, in contrast to the starting strain, was no longer sensitive to maltose. Even though the pink color suggested a weak Mal$^+$ phenotype, the plasmid did not allow growth on minimal maltose plates. pRP10, the plasmid isolated from this transformant, gave rise to the same phenotype after retransformation into CB39, indicating that the phenotype was due solely to the cloned gene. The restriction analysis of pRP10 is shown in Fig. 1. The partial DNA sequence of the insert showed 100% identity to a region at 10.8 min on the *E. coli* chromosome containing two complete open reading frames, *hemH* (*visA*) and *orf203*, both of which had been sequenced previously (28). The section also contained the 5′ and 3′ (respectively) truncated portions of *adk* (12) and *gsk* (19) adjacent to *hemH* and *orf203* (Fig. 1). To determine which gene was responsible for the observed phenotype, most of *hemH* was deleted (Fig. 1). The resulting plasmid, pRP11, still caused the same phenotype as pRP10, suggesting that *orf203* is the causative gene. Moreover, plasmid pRP12 harboring a Cam^r insertion in *orf203* no longer elicited the observed phenotype.

***orf203* (*aes*) encodes an enzyme with esterase activity.** In contrast to our expectation, extracts of strains harboring plasmid-encoded *orf203* did not contain maltose phosphorylase activity. They also did not hydrolyze maltose to glucose. The sequence of *orf203* as deposited in SWISSPROT (accession no. P23872) revealed homology to the genes encoding lipases and esterases (28). Since the amino acid homology did not extend over the entire length of the deduced amino acid sequence and since the introduction of a frame shift produced more extended homology (3, 9), we resequenced *aes*. The sequence is shown in Fig. 2. The change of base 1048 from T to G did not result in an alteration of the deduced amino acid sequence, but the addition of C at position 1040 resulted in a frame shift causing an elongation of the previously published sequence of *orf203* (28) by 37 amino acids at the C terminus. The same shift in the open reading frame had been found in more recent GenBank submissions of *orf203* (*ybaC*) (accession no. AE000153 and U82664).

To determine the transcriptional start point of *aes*, we used the technique of primer extension of isolated RNA by reverse transcriptase. This is shown in Fig. 3. Accordingly, the transcriptional start is at position 142, as depicted in Fig. 2. The corresponding −10 and −35 putative promoter sequences do not show homology to the consensus sequence. The coding sequence of *aes* starts with ATG at base 220 and ends at the stop codon TAG at base 1177 of the sequence shown in Fig. 2;

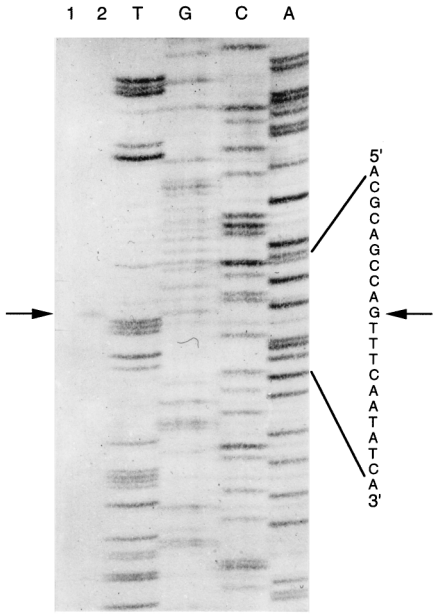

FIG. 3. Transcriptional start point of *aes*. Primer extension products with a digoxygenated 22-mer primer and mRNA from MC4100 harboring pBR322 (lane 1) and MC4100 harboring pRP11 (lane 2) are shown. For localization of the product, a sizing ladder indicated by T, G, C, and A (with the same primer) is also shown. The arrow indicates the transcriptional start point of *aes*. The sequence on the right represents the noncoding strand.

it encodes a protein of 319 amino acids with a calculated molecular weight of 36,034. When the deduced amino acid sequence was compared with that of an esterase from *Acinetobacter calcoaceticus* (34) or a lipase from a *Moraxella* sp. (17) (Fig. 4), 27.6 and 24.4% identity, respectively, was observed over the entire length of the protein including the "GDSXG" sequence (at positions 163 to 167 of the sequence shown in Fig. 4) typical of lipolytic enzymes (4). To test for esterase activity, *p*-nitrophenyl acetate (PNPA) at saturating concentrations was used in extracts of a strain harboring pRP11. The data are shown in Table 2. PNPA-hydrolyzing activity was increased about fivefold in strain BRE1162 harboring pPR11 in comparison to the same strain harboring vector pBR322 only. Therefore, we renamed *orf203* as *aes* (for acetyl esterase). The PNPA-hydrolyzing activity was not further decreased in derivatives of MC4100, our control laboratory strain, carrying a chromosomal *aes*::Cam^r insertion or an *aes*::*lacZ* fusion, indicating that *aes*-encoded activity is not the only esterase activity in MC4100. The *aes* gene was cloned under an IPTG-inducible promoter (pAS1). Strains harboring this plasmid were able to grow on plates containing 0.5% triacetyl glycerol (triacetin) as the sole carbon source (34) only in the presence of IPTG, indicating that triacetin is a substrate of Aes.

Expression of *aes* in the minicell system. The minicell-producing strain DS410-T (26, 35) was transformed with pRP11 harboring the intact *aes* gene, with pRP12 harboring *aes*::Cam^r, or with the control plasmid pBR322 alone. The minicells were isolated and labeled with [^{35}S]methionine, and the labeled proteins were analyzed by SDS-PAGE followed by autoradiography (Fig. 5). pRP11 gave rise to the synthesis of a protein of 37 kDa that was absent in either pRP12- or pBR322-carrying cells. Therefore, this protein represents the gene product of *aes*. Curiously, by this technique, the protein always appeared as a closely spaced double band where the slower-migrating band is less intense. Since this was reminiscent of secreted

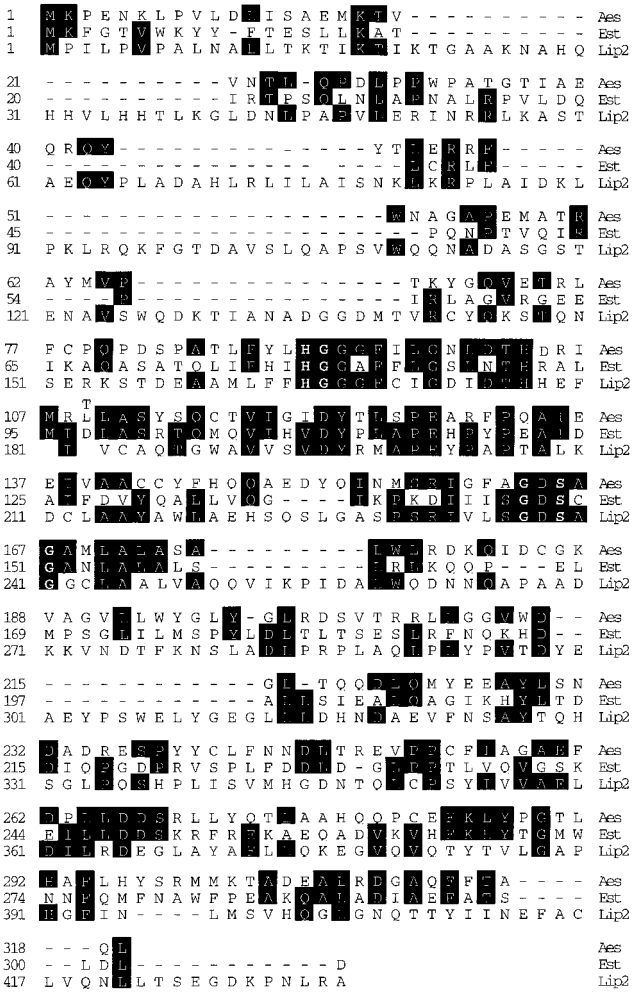

FIG. 4. Homology of Aes to lipases and esterases. The alignment of Aes with an esterase from *A. calcoaceticus* (Est) (43) (SWISSPROT accession no. P18773) (27.6% identity) and with a lipase from *Moxarella* TA144 (Lip2) (17) (SWISSPROT accession no. P24484) (24% identity) is shown. Identical amino acids are in black boxes. The GDSXG sequence, conserved in lipolytic enzymes, is at positions 163 to 167 of the Aes sequence. Boldface letters correspond to amino acids conserved in amino-terminal domains of some lipases (17).

proteins, we tested the release of PNPA-hydrolyzing activity in the periplasmic fraction after applying the classical cold osmotic shock procedure (29) to cells harboring pRP11. More than 97% of the total esterase activity was found in the soluble cytoplasmic fraction, and the protein would therefore not be classified as a secreted protein. Also, the deduced amino acid sequence of *aes* did not show the characteristic pattern of a cleavable signal sequence at its N terminus.

TABLE 2. PNPA-hydrolyzing activity of different strains

Strain	Relevant genotype	Activity[a]
BRE1162(pBR322)	*aes*$^+$	0.050
BRE1162(pPR11)	*aes*$^+$ p*aes*$^+$	0.260
MC4100	*aes*$^+$	0.050
RP104	*aes*::cam65	0.042
RP105	*aes*::*lacZ*	0.046

[a] Activity is given in units per milligram of protein in cell extracts of strains grown at 37°C.

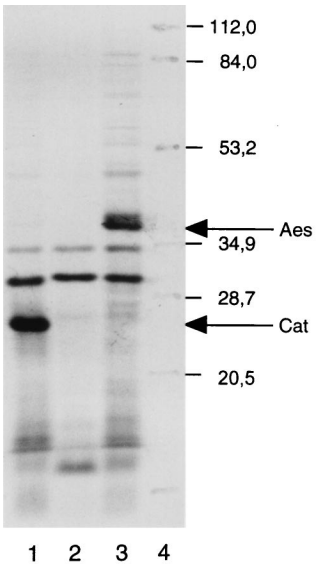

FIG. 5. Expression of *aes* in the minicell system. Minicells of plasmid-harboring cells were labeled with [^{35}S]methionine, and the proteins synthesized were analyzed by SDS-PAGE followed by autoradiography. Lanes: 1, plasmid pRP12 (*aes*::cat); 2, pBR322 (vector control); 3, plasmid pRP11 (*aes*$^+$); 4, protein standard with molecular masses given in kilodaltons. Aes and Cat indicate the positions of the Aes protein and chloramphenicol transacetylase, respectively.

The expression of *aes* is temperature sensitive and deregulated when plasmid encoded. The *aes*::*lacZ* fusion was used to study the expression of *aes* under different growth conditions, at different temperatures, and plasmid as well as chromosomally encoded. The fusion represents a hybrid protein where β-galactosidase is inserted after amino acid 65 of Aes. Enzyme activity was tested in cells grown overnight. The growth phase had no significant effect on *aes* expression. Table 3 shows that the degree of *aes* expression did not depend significantly on the carbon source used. However, the expression was temperature sensitive, being reduced 10-fold after growth at 42°C in comparison to growth at 37°C. This effect could also be seen when *aes* was plasmid encoded. The expression of plasmid-encoded *aes* was surprisingly high, indicating the titration of a repressor. However, the presence of plasmid-encoded *aes*::Cam^r still containing the control region of *aes* and thus allowing the titration of a potential repressor had no effect on the expression of chromosomally encoded *aes*::*lacZ*.

TABLE 3. Expression of *aes*::*lacZ* under different growth conditions

Strain	Relevant genotype	Growth medium[a]	Enzyme activity[b] at growth temperature of:		
			28°C	37°C	42°C
RP105	*aes*::*lacZ*	MMA-Gly	0.0025	0.0020	0.0003
	aes::*lacZ*	MMA-Mal	0.0016	0.0020	0.0002
	aes::*lacZ*	LB broth	0.0042	0.0031	0.0003
	aes::*lacZ* p*aes*::Cam^r	LB broth	0.0041	0.0035	0.0003
MC4100(pRP13)	p*aes*::*lacZ*	MMA-Gly	1.68	1.87	ND[c]
	p*aes*::*lacZ*	MMA-Mal	1.88	1.75	ND
	p*aes*::*lacZ*	LB broth	2.00	1.98	0.22

[a] Gly, glycerol; Mal, maltose.
[b] Enzyme activity is given in units per milligram of protein.
[c] ND, not determined.

TABLE 4. Overexpression of *aes* reduces *mal* gene expression as measured by maltose transport activity[a]

Strain	Relevant genotype	Transport activity with carbon source of:	
		Glycerol	Glycerol plus maltose
MC4100(pBR322)	*mal*$^+$	5.0	97.1
MC4100(pRP11)	*mal*$^+$ p*aes*$^+$	0.2	0.4
CB39(pBR322)	*malQ*::Tn*10*	48.1	81.1
CB39(pRP11)	*malQ*::Tn*10* p*aes*$^+$	8.4	17.4
JB3018(pBR322)	*malT*(Con)	132.4	133.7
JB3018(pRP11)	*malT*(Con) p*aes*$^+$	178.3	187.2

[a] Transport activity was measured at 52 nM substrate concentration and is given as picomoles of [^{14}C]maltose taken up per 10^9 cells per minute.

The presence of plasmid-encoded *aes* results in the repression of the maltose system. Since *aes* did not encode an enzyme with maltose phosphorylase activity, we searched for another reason why overexpression of *aes* abolished the maltose sensitivity of a *malQ*::Tn*10* strain. We tested whether the overexpression of *aes* affected the expression of the maltose system genes. Maltose transport activity was chosen as a measure. The results are shown in Table 4. Maltose transport was severely repressed in a *mal*$^+$ strain after growth in the presence and absence of maltose. In the *malQ*::Tn*10* strain CB39, constitutive maltose transport was reduced by a factor of 4 in the presence of the plasmid. The overexpression of *aes* has no effect on the constitutivity in a *malT*(Con) strain. Overexpression of *aes* specifically affected the maltose system. Similar assays with the *phoB*-dependent G3P-specific Ugp system (13, 30) or the galactose-specific Mgl system (6, 18, 21) showed no effect of plasmid-encoded *aes* (data not shown). Both systems represent binding protein-dependent ATB-binding cassette transport systems (7) similar to the maltose transport system.

The overexpression of *aes* reduced the transcription of *malK* and *malE*, the first genes in the two divergently oriented transport operons, more than 10-fold but did not affect the expression of *malT*. This was measured by using transcriptional β-galactosidase fusions and is shown in Table 5. However, the loss of *aes* function did not increase *malK* or *malE* expression.

Repression by Aes is counteracted by elevated levels of MalT. The *aes* gene was cloned under an IPTG-inducible promoter (pAS1). The presence of IPTG induced the synthesis of a 37 kD protein that was visible in SDS-PAGE (Fig. 6). The strain (BRE1162) contains a *malK-lacZ* fusion and is thus constitutive for the expression of the remaining maltose genes

TABLE 5. Overexpression of *aes* reduces the expression of *malK*::*lacZ* and *malE*::*lacZ*, but not *malT*::*lacZ*

Strain	Relevant genotype	β-Galactosidase activity (U/mg of protein)
RP110	*malT*::*lacZ aes*::Cam^r	0.38
BRE1161(pBR322)	*malT*::*lacZ*	0.29
BRE1161(pRP11)	*malT*::*lacZ* p*aes*$^+$	0.49
RP111	*malK*::*lacZ aes*::Cam^r	1.55
BRE1162(pBR322)	*malK*::*lacZ*	1.41
BRE1162(pRP11)	*malK*::*lacZ* p*aes*$^+$	0.15
RP112	*malE*::*lacZ aes*::Cam^r	0.61
BRE1163(pBR322)	*malE*::*lacZ*	0.48
BRE1163(pRP11)	*malE*::*lacZ* p*aes*$^+$	0.02

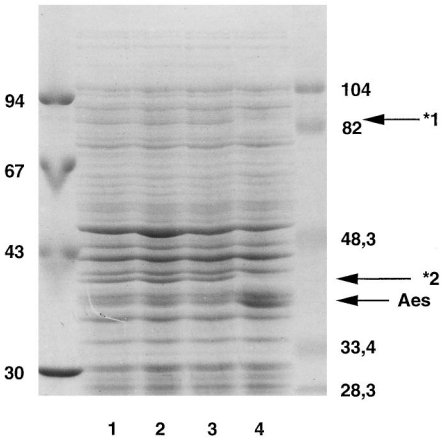

FIG. 6. SDS-PAGE of cells overproducing Aes. Strain BRE1162 (*malK-lacZ*) harboring different plasmids was grown in NZA medium overnight. Equal amounts of cells (about 50 μg of total protein) were dissolved in sample buffer and subjected to SDS-PAGE. The following plasmids were used: lane 1, pGDR11 (vector for *aes*); lane 2, same as lane 1 but grown with 1 mM IPTG; lane 3, pAS1 (p*aes*$^+$); lane 4, same as lane 3 but grown with 1 mM IPTG. *1 and *2 indicate the presumptive positions of MalP and MalE. The numbers on the left and right side of the gel show the positions of marker proteins in kilodaltons.

such as MalE (encoding maltose-binding protein) and MalP (encoding maltodextrin phosphorylase). Two protein bands corresponding to the molecular weights of MalE and MalP disappeared after the induction of Aes (Fig. 6). By using specific antibodies against MalE, the identity of the first band (*2 in Fig. 6) to MalE could be confirmed by Western blotting. In addition, by using the same technique, the reduction of MalS (a MalT-dependent α-amylase) after Aes induction could be demonstrated (data not shown). Repression of *malK-lacZ* by pRP11 was strongly reduced by the additional presence of the *malT*$^+$-harboring plasmid pRP31: the latter plasmid caused a strong increase in the expression of *malK-lacZ* by itself or in the presence of uninduced Aes (pAS1). However, IPTG-induction of Aes again reduced the stimulating effect of elevated

TABLE 6. Aes-mediated repression of *malK-lacZ* is counteracted by increasing MalT

Plasmid(s)	Relevant genotype[a]	β-Galactose activity (U/mg of protein)[b]	
		Uninduced	IPTG induced
pBR322	Vector for *aes*$^+$ (wt pr.)	0.881	0.903
pRP11	*aes*$^+$ (wt pr.)	0.115	0.115
pHSG575	Vector for p*malT*$^+$	1.753	1.7
pRP31	p*malT*$^+$	5.873	5.612
pRP11 + pHSG575	*aes*$^+$ (wt pr.) + vector for p*malT*$^+$	0.335	0.338
pRP11 + pRP31	*aes*$^+$ (wt pr.) + p*malT*$^+$	2.583	2.188
pGDR11	Vector for p*aes*$^+$ (ind. pr.)	1.398	1.659
pAS1 (Aes)	*aes*$^+$ (ind. pr.)	0.777	0.151
pAS1 + pHSG575	*aes*$^+$ (ind. pr.) + vector for p*malT*$^+$	1.14	0.549
pAS1 + pRP31	*aes*$^+$ (ind. pr.) + p*malT*$^+$	6.358	1.161

[a] wt pr., wild-type promoter; ind. pr., IPTG-inducible promoter.
[b] The specific β-galactosidase activity of strain BRE1162 (*malK-lacZ*), harboring different plasmids, after growth overnight in MMA and glycerol as the carbon source is given.

MalT levels (Table 6). This demonstrates that the degree of repression is dependent on the concentration of Aes and that it can be counteracted by increasing the level of MalT. The effect of plasmid-encoded *aes* on *mal* gene expression is reminiscent of similar effects caused by the overexpression of MalK (37) and MalY (47). Indeed, we observed that the overproduction of MalK and MalY also resulted in the loss of maltose sensitivity exhibited by a *malQ*::Tn*10* strain when plated on MacConkey maltose plates.

DISCUSSION

In this paper, we report the characterization of *aes*, which encodes an enzyme with esterase activity. The deduced amino acid sequence of the encoded protein shows significant homology to lipases containing the GDSXG signature typical of lipolytic enzymes. The function of this protein in *E. coli* is unknown, and insertion mutations in *aes* do not encode an easily discernable phenotype. Plates containing triacetyl glycerol as the only carbon source allow the growth of strains that overproduce Aes, indicating that this protein functions as an esterase. However, enzyme assays (both radioactive and pH-Stat) for lipase and phospholipase activity done by Allen Place (Center of Marine Biotechnology, Baltimore, Md.) gave no indication of Aes being an enzyme with lipase activity. From the isolation procedure, it is clear that the enzyme is a typical soluble cytoplasmic protein. The classical osmotic shock procedure identifying periplasmic proteins (29) did not release Aes activity, consistent with the fact that the *N*-terminal sequence does not contain a discernible signal sequence for secretory proteins. Aside from the observation that the expression of *aes* is temperature sensitive, it appears to be expressed constitutively.

The overexpression of plasmid-encoded *aes* led to strong repression of several *mal* genes tested without affecting the expression of *malT*, which encodes the positive *mal* gene activator. However, an increase in the level of MalT counteracts the effect of Aes. This indicates that Aes interacts directly with MalT, rendering it inactive as a transcriptional activator. The effect of Aes is reminiscent of the effect which overproduction of two other proteins has on *mal* gene expression, i.e., MalK, the subunit of the binding protein-dependent ATP-binding cassette transporter, itself a *mal* gene-encoded protein (24, 37), and MalY (36), an enzyme exhibiting cystathionase activity (47). Even though the strength of repression with the three proteins are not identical, the patterns are very similar. For instance, all of them show very little effect in *malT*(Con) strains where the induction of the *mal* system becomes independent of inducer or under conditions where the level of MalT is increased. In the case of MalY, it is clear that the enzymatic activity (cystathionase) is unrelated to the repression of *mal* gene expression, since there exists a mutant that has lost cystathionase activity but has retained full repression activity (47). Similarly, in MalK, the domain responsible for repression can be separated from its transport activity (24). We speculate that a structural motif present in all three proteins, but not recognizable by sequence, interacts with MalT, thus inactivating the function of MalT as a transcriptional activator.

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