Growth Defects of *Escherichia coli* Cells which Contain the Gene of an α -Amylase from *Bacillus coagulans* on a Multicopy Plasmid

By KARINE WILLEMOT^{1,2*} AND PIERRE CORNELIS²

¹ Département de Biologie Moléculaire, Unité de Génétique Moléculaire, Institut Pasteur, F-75724 Paris cedex 15, France

² Laboratoire de Cytogénétique, Institut Carnoy Université de Louvain, B-1348 Louvain-la-Neuve, Belgium

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An α -amylase gene from *Bacillus coagulans* has previously been cloned in *Escherichia coli* and shown to direct the synthesis of an enzymically active protein of 60000 Dal (Cornelis *et al.*, 1982). In one particular *E. coli* host, strain HB101, amylase was found to accumulate in the periplasmic space. To study the processing and the location of the amylase, plasmid pAMY2 was introduced into *E. coli* 188 which is a strain constitutive for alkaline phosphatase, a periplasmic marker, and for β -galactosidase, a cytoplasmic marker. Abnormally large amounts of both α -amylase and β -galactosidase were found in the culture fluid of cells grown in rich medium. Furthermore a severe growth defect was found when cells containing pAMY2 were grown in maltose and glycerol media, while the ability to grow on glucose remained normal. This defect could be reversed by two types of spontaneous mutations. Mutations in the first class are located on the plasmid and correspond to the insertional inactivation of the amylase gene by IS1. Mutations in the second class are located on the host chromosome. These results suggest that the synthesis and export of *B. coagulans* α -amylase is deleterious to *E. coli*, especially in media containing maltose or glycerol as sole carbon source.

INTRODUCTION

A great number of species belonging to the genus *Bacillus* synthesize one or several extracellular amylases (Priest, 1977). *Bacillus coagulans* produces a thermostable α -amylase, the gene of which was cloned and found to be expressed at a high level in *Escherichia coli* HB101 (Cornelis *et al.*, 1982). The recombinant plasmid, called pAMY2, contained a cloned *EcoRI* fragment of 3.31 kb that directed the synthesis of a liquefying α -amylase of molecular weight 60000. The high level of expression of this particular amylase in *E. coli* was surprising since genes from Gram-positive bacteria are generally expressed at a low rate in *E. coli*. In particular, this was the case for *Bacillus licheniformis* β -lactamase (Gray & Chang, 1981; Brammar *et al.*, 1980) and for other amylase genes previously cloned in our laboratory (Colson *et al.*, 1981). The presence of large amounts of amylase made it easier for us to study the location of the enzyme in *E. coli*.

An interesting aspect that could be investigated was the fate of a Gram-positive extracellular protein in a Gram-negative host where there is both an inner and an outer membrane, separated by the periplasmic space (Rogers *et al.*, 1980). Some eukaryotic extracellular proteins such as chicken ovalbumin or human insulin, when synthesized in *E. coli*, were previously shown to cross the inner membrane but to be blocked in the periplasmic space (Baty *et al.*, 1981; Talmadge *et al.*, 1981). Gray & Chang (1981) found that the *B. licheniformis* β -lactamase also remains in the periplasmic space. Preliminary experiments with *B. coagulans* α -amylase

* Requests for offprints should be sent to this author at the Belgium address.

indicated that the enzyme is located mainly in the periplasm of *E. coli* K12 HB101 while some extracellular activity was also found (Cornelis *et al.*, 1982). We tried to locate the enzyme by studying the expression of the amylase coded by pAMY2 in *E. coli* K12 188, a strain constitutive for alkaline phosphatase, a periplasmic marker, and for β -galactosidase, a cytoplasmic marker. However, difficulties arose because of an unexpected distribution of amylase and β -galactosidase. In the course of the study we found defects in growth on some minimal media and the results presented in the present paper suggest that the synthesis of large amounts of α -amylase is responsible for this deleterious effect.

METHODS

Strains, media and genetic techniques. The bacterial strains, bacteriophages and plasmids used are listed in Table 1.

Rich medium (LB) and minimal medium (M63) have been described previously (Miller, 1972*a*). To grow cells harbouring pBR322 or pAMY2, solid media were supplemented with 30 μ g ampicillin ml⁻¹ and liquid media with 50 μ g ampicillin ml⁻¹. For the detection of amylase activity on plates, minimal media contained 0.4% (w/v) soluble starch (Corn Products Company, Vilvoorde, Belgium).

General cloning procedures. Extraction of plasmid DNA, restriction, and analysis of the fragments by agarose gel electrophoresis have been described previously (Cornelis *et al.*, 1982). Transformation was performed as described by Lederberg & Cohen (1974).

Osmotic shock. The specific extraction of periplasmic proteins was carried out as described by Neu & Heppel (1965).

Protein determination and enzyme assay. Protein concentration was estimated by the Lowry method. β -Galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl- β -D-galactoside as described by Miller (1972*b*). One unit of β -galactosidase activity is defined as the amount of enzyme producing 1 nmol *o*-nitrophenol min⁻¹ at 28 °C. Alkaline phosphatase activity was assayed by hydrolysis of *p*-nitrophenyl phosphate (Garen & Levinthal, 1960). One unit of alkaline phosphatase activity is defined as the amount of enzyme producing 1 nmol *p*-nitrophenol min⁻¹ at 37 °C. Assay of amylase activity was as described by Cornelis *et al.* (1982). One unit of amylase activity is defined as the amount of enzyme producing 1 mg reducing sugars min⁻¹ at 50 °C, taking maltose as the reference sugar.

The uptake of [¹⁴C]maltose by exponentially-growing cells was measured as previously described (Szmecman *et al.*, 1976).

Separation of proteins by polyacrylamide gel electrophoresis. Proteins were separated on SDS-polyacrylamide gels according to Laemmli (1970). Samples were run on 10 to 20% SDS-polyacrylamide gradients.

Isolation of mutants able to grow normally on maltose. A fully grown culture of strain 188(pAMY2) from minimal maltose medium was plated on to similar solid medium. After 2 d incubation at 37 °C bigger colonies protruding from the bacterial lawn could be seen and were picked with a tooth pick under the binocular microscope and streaked three times to give single colonies. A total of 100 such individual clones were tested for their ability to grow on maltose and for amylase activity.

Plasmid curing. Plasmid curing was obtained after five successive passages of strain 188(pAMY2) in LB medium in the absence of antibiotics. The last culture was plated on to the same medium and isolated colonies were tested for sensitivity to ampicillin and tetracycline. About 5% of the clones were found to be sensitive to the antibiotics, and were therefore assumed to have lost the plasmid.

Table 1. *Bacterial strains, plasmids and bacteriophage*

Material	Genotype/properties	Origin
<i>E. coli</i> K12 strains		
188	<i>metB thi lacI pho(S,T)</i>	Lazzaroni & Portalier (1981)
HB101	<i>hsdS leu pro lac gal</i> <i>thi strA recA</i>	Boyer & Roulland-Dussoix (1969)
DS410	(F ⁻) <i>ara azi tonA lacY</i> <i>minA minB rpsL malA</i> <i>xyl mtl thi</i>	Reeve (1977)
Plasmids		
pBR322	Amp ^R Tet ^R	Bolivar <i>et al.</i> (1977)
pAMY2	Amp ^R Tet ^R Amy ⁺	Cornelis <i>et al.</i> (1982)
Bacteriophage		
λ R14(IS1)		Brachet <i>et al.</i> (1970)

Fig. 1. Kinetics of (a) α -amylase, (b) β -galactosidase and (c) alkaline phosphatase production by strain 188(pBR322) (---) and strain 188(pAMY2) (—). ●, Total activity; □, periplasmic activity; ○, extracellular activity; and ■, cellular activity. Bacteria were grown at 37 °C in LB medium.

Nick-translation of λ R14(IS1) and hybridization. Nick-translation with [³²P]dCTP was performed with the New England Nuclear 'nick-translation kit'. The different plasmid DNAs were prepared according to Birnboim & Doly (1980) and digested with *Pst*I. The fragments were separated on an agarose gel (Sharp *et al.*, 1973). The transfer to nitrocellulose filters ('Genescreen'; New England Nuclear) and hybridization were performed according to Southern (1979).

Purification and labelling of minicells. The method described by Reeve (1977) was used. The minicells produced by strain DS410 were labelled in Hershey's minimal medium (Worcel & Burgi, 1974) for 1 h at 37 °C, with 50 μ Ci (185 kBq) L-[³⁵S]methionine (Amersham; 1.05×10^3 Ci mmol⁻¹, 38.85 TBq mmol⁻¹).

RESULTS

Location of the α -amylase in *E. coli* 188

The synthesis of α -amylase in *E. coli* HB101(pAMY2) was followed during growth in LB medium and its cellular location was studied. As previously found, about 80% of the activity was periplasmic (Cornelis *et al.*, 1982). In order to compare the cellular distribution of amylase with that of typical cytoplasmic and periplasmic enzymes, this experiment was repeated after transfer of plasmid pAMY2 into *E. coli* 188. In contrast to the results obtained with HB101(pAMY2) we found that after the osmotic shock only 48% of the amylase activity was in the periplasm, with 48% in the extracellular fraction and 4% cell-bound (Fig. 1a). The synthesis of β -galactosidase and alkaline phosphatase was also followed for both strain 188(pBR322) and strain 188(pAMY2). Abnormally high activities of β -galactosidase (4900 units ml⁻¹ from a total of 34000 units ml⁻¹ in the stationary phase of growth) were found in the extracellular fraction of strain 188(pAMY2) while the same enzyme was found exclusively in the cellular fraction in strain 188(pBR322) (Fig. 1b, c). The synthesis of amylase seems to perturb the distribution of both β -galactosidase and the amylase itself in this particular host.

Inhibition of cell growth by plasmid pAMY2 and isolation of mutants with normal growth

The growth rates of strain 188(pBR322) and strain 188(pAMY2) in different media are given in Table 2. The generation times were the same for strain 188(pBR322) and strain 188(pAMY2) in LB medium and very similar in minimal glucose medium. However, strain 188(pAMY2) grew more slowly than strain 188(pBR322) in maltose and glycerol media. This growth defect on

Table 2. *Generation times of strain 188(pBR322), strain 188(pAMY2) and different mutants in various media*

Sugars were added at a concentration of 0.4% (w/v) to M63 medium.

Strain	Generation time (min)			
	LB medium	Glucose medium	Maltose medium	Glycerol medium
188(pBR322)	35	85	90	90
188(pAMY2)	35	100	180	180
KW5	35	85	90	90
KW6	35	85	120	120
KW7	35	85	90	90

maltose and glycerol was also found for other *E. coli* strains harbouring pAMY2, including HB101 (results not shown). When melibiose, mannitol, arabinose, fructose, lactose and galactose were tested, slight growth defects were found for melibiose and mannitol (results not shown).

Spontaneous mutants growing faster on maltose (and also on glycerol) were isolated as described in Methods, and their ability to produce amylase was tested *in situ* on solid medium containing soluble starch. The mutants could be divided into two classes: those without detectable amylase activity (Amy⁻) and those with detectable amylase activity (Amy⁺). All Amy⁻ mutants grew normally on glycerol as well as on maltose. Some Amy⁺ mutants such as strain KW5 grew normally on maltose but others, such as strain KW6, were still somewhat inhibited (Table 2). The mutants with intermediate ability to grow on maltose produced amylase at a level approaching that found for strain 188(pAMY2) while the mutants which grew as well as strain 188(pBR322) produced much less amylase. This result suggested that the synthesis of amylase might be responsible for the inhibition of growth on both maltose and glycerol.

Analysis of the Amy⁻ mutants

Nine of the mutants which lacked amylase activity according to the plate test were further analysed. They were first checked for amylase production in liquid culture. No trace of activity was found, either in the growth medium or in the cells (results not shown).

The plasmid DNA extracted from the mutants was used to transform *E. coli* 188. All transformants grew normally on maltose minimal medium and failed to produce amylase; therefore, we concluded that the Amy⁻ phenotype resulted from an alteration on the plasmid.

The restriction patterns obtained with *Pst*I for nine of these plasmids identified four types of mutants, designated A, B, C and D (Fig. 2a). In addition to the *Pst*I site of pBR322, plasmid pAMY2 contains two additional *Pst*I sites giving a total of three *Pst*I fragments of 4.65 kb, 2.20 kb and 0.85 kb, respectively. Mutants belonging to class A have four sites for *Pst*I giving four fragments of 4.65 kb, 2.54 kb, 0.85 kb and 0.46 kb, respectively. These mutants have an insertion sequence of 0.8 kb (Fig. 3). The insertion is in the amylase gene since the coding sequence of the amylase gene in pAMY2 has been shown to be located between the *Pst*I site at coordinate 2.25 and the *Sac*I site at coordinate 0.7 (Cornelis *et al.*, 1982). This insertion has an additional *Pst*I site, located as shown. Mutants belonging to class B have a plasmid which is cut by *Pst*I into four fragments of 4.65 kb, 1.90 kb, 1.02 kb and 0.85 kb, respectively; this plasmid probably has the same insertion sequence at the same site, or near the same site, as the plasmid of class A but in the opposite orientation (Fig. 3). Plasmids of mutants belonging to class C are cut by *Pst*I into four fragments of 4.65 kb, 2.90 kb, 0.85 kb, respectively, plus at least one hypothetical 0.20 kb fragment not seen on the gel. The plasmid contains an insertion of 0.8 kb with one *Pst*I site in the structural part of the amylase gene near the second *Pst*I site of pAMY2. Mutants belonging to class D have an insertion of 1.3 kb without a *Pst*I site. Both the size of insertion found in the classes of plasmids A, B and C and the presence of one *Pst*I site near one of its extremities were indications that this insertion could be IS1 (Ohtsubo & Ohtsubo, 1978).

Fig. 2. Agarose gel electrophoresis and filter hybridization of fragments of plasmid DNA. (a) The 1.0% (w/v) agarose gel containing restricted DNA. (b) The autoradiogram resulting from the transfer of the fragments from the agarose gel to nitrocellulose filter paper and annealing to ^{32}P -labelled $\lambda\text{R14}(\text{IS1})$ DNA. Lane 1, molecular weight markers of λDNA digested with *Hind*III; lane 2, pBR322 digested with *Pst*I; lane 3, pAMY2 digested with *Pst*I; lanes 4–7, *Pst*I digests of plasmid DNA from the following classes of mutants: lane 4, class C; lane 5, class A; lane 6, class D; and lane 7, class B. The different mutants of each class gave the same restriction pattern. One of the nine isolated mutants belongs to class A, two to class B, four to class C and two to class D. For practical reasons, the strips of nitrocellulose paper were of such size that the 0.45 kb fragment is absent from the autoradiogram (lane 5).

To check this hypothesis, the fragments from the gel (Fig. 2a) were transferred by blotting to a nitrocellulose filter (Southern, 1979) and the filter was hybridized with ^{32}P nick-translated DNA from $\lambda\text{R14}(\text{IS1})$. The autoradiogram shows that IS1 hybridized in all cases, even in the case of the plasmid of class D which was shown previously to contain an insertion of 1.30 kb without a *Pst*I site (Fig. 2b). However, this class of mutants (D) was not analysed further at this stage.

The different plasmids containing IS1 were introduced into strain DS410 in order to study the products directed by the plasmid in minicells (Reeve, 1977). An autoradiogram of the proteins made in minicells containing pBR322, pAMY23 and the plasmids of class A, B, C and D is shown in Fig. 4. pAMY23 is a derivative of pAMY2 containing the large *Eco*RI–*Hind*III insert of pAMY2; this derivative was used instead of pAMY2 because of the high instability of the latter plasmid in DS410. pAMY23 contains an intact amylase gene and directs the synthesis of two large proteins, one appearing as a faint band of 62 000 Dal, and one major band of 60 000 Dal which has been shown previously to correspond to α -amylase (Cornelis *et al.*, 1982). We assume that the band of 62 000 Dal represents the precursor of α -amylase, still containing the leader sequence. A new protein of 26 500 Dal is made in minicells containing the plasmids of class A, B, C and D. This protein could be encoded between the *Hind*III and the *Eco*RI sites on the *B. coagulans* DNA in pAMY2; this portion of DNA is absent in pAMY23 which explains the absence of the protein in lane 1. The fact that no other protein of molecular weight higher than 20 000 Dal is present in lanes 2–5 (Fig. 4) and that no difference exists between the different mutants confirms that in all cases IS1 prevents expression of the amylase gene.

Analysis of the *Amy*⁺ mutants

The different *Amy*⁺ mutants, as noted above, produced different amounts of amylase. Quantitative assays performed after growth in liquid media demonstrated that some, such as

Fig. 3. Restriction map of the plasmids pAMY2 and the mutants of classes A, B and C which have inserted an IS1. The arrow indicates the direction of transcription of the α -amylase gene, the continuous bold lines represent the IS1 insert, the bold dashed lines represent *B. coagulans* DNA, and the thin lines represent pBR322 DNA.

strain KW6, produced the same level of amylase as strain 188(pAMY2) while others, such as strain KW5, produced less amylase (Table 3).

Plasmids were extracted from cells of 10 independent mutants producing different amounts of amylase. These plasmids were reintroduced into strain 188. All of the transformants showed a much reduced ability to grow on maltose and produced the same amount of amylase as strain 188(pAMY2) (data not shown). Conversely, when the mutants were cured of their plasmids and then transformed again with the original plasmid, pAMY2, the transformants showed the same mutant phenotype as before curing. These results suggested that the mutations are on the bacterial chromosome.

Amylase production and maltose transport

The analysis of the mutants indicated that the growth inhibition on maltose and glycerol is linked with the synthesis of amylase since all the mutants growing normally on maltose and

Fig. 4. Autoradiogram of [^{35}S]methionine-labelled proteins synthesized in minicells of strain DS410 containing the following plasmids. Lane 1, pAMY23; lanes 2–5, plasmid DNA from Amy $^{-}$ mutants of the following classes: lane 2, class C; lane 3, class A; lane 4, class D; lane 5, class B. Lane 7, no plasmid; lane 8, pBR322. Lane 6 contains molecular weight standards. Electrophoresis was performed in a 10 to 20% polyacrylamide gradient gel (Laemmli, 1970).

Table 3. *Amylase production in different strains grown in LB medium and in minimal glycerol medium*

Strain	Amylase activity [units (mg protein) $^{-1}$]	
	LB medium	M63 glycerol medium
188(pAMY2)	1.57	1.44
KW5	0.35	0.37
KW6	1.49	1.42
KW7	0.63	ND
KW8	1.07	ND

ND, Not done.

glycerol were impaired in their ability to produce amylase. It could be postulated that the inhibition of growth, significant only on maltose and glycerol media, resulted from increased amylase production in these media. The results in Table 3 show that the activity of amylase was the same whether the cells were grown on LB medium or on minimal glycerol medium. Amylase activity could not be assayed in maltose-containing media. Another possibility was that amylase production competed at the level of protein export. The presence of large quantities of amylase crossing the inner membrane could reduce the export of the periplasmic and membrane proteins necessary for the transport of metabolites. Determination of the rate of maltose transport

supported this hypothesis. The rate of maltose transport was indeed reduced by a factor of two in strain 188(pAMY2) compared to strain 188(pBR322) (4.5 nmol compared to 9.3 nmol [^{14}C]maltose transported per 10^9 bacteria min^{-1}). Intermediate rates were observed for the mutants with capacity to produce amylase and, as expected, the rate was inversely proportional to the amount of amylase produced.

DISCUSSION

The first results concerning the expression of the *B. coagulans* α -amylase in *E. coli* HB101 indicated that large amounts of periplasmic amylase were produced and that the synthesis of this foreign protein did not interfere with the growth in LB medium. The transfer of plasmid pAMY2 into a different host, *E. coli* 188, to study more accurately the cellular location of amylase, revealed that amylase as well as β -galactosidase, a cytoplasmic marker, leaked into the medium but that alkaline phosphatase, a periplasmic marker, did not. Furthermore, growth was found to be inhibited on some media, this phenomenon being true for strains 188 and HB101. The leakage of the amylase into the medium was found only when pAMY2 was in strain 188, suggesting that the constitutive synthesis of alkaline phosphatase or β -galactosidase might be responsible for this phenomenon.

The growth inhibition observed on maltose- or glycerol-containing media appears to be a consequence of amylase synthesis rather than a consequence of some other genetic information carried by the plasmid. Indeed, all of the mutations which permitted strain 188(pAMY2) to grow on maltose or glycerol simultaneously affected amylase synthesis. Some of the mutations correspond to the insertion of an IS1 sequence in the plasmid-borne gene of α -amylase, while others were located on the chromosome and affected, to different extents, the expression of the α -amylase gene.

Two main conclusions can be drawn from this work. One concerns the difficulties inherent in the cloning of genes encoding extracytoplasmic proteins. Others have already noted that the cloning of such genes is sometimes impossible, presumably because it leads to cell death (Henning *et al.*, 1979). What we have shown here is that, even when this cloning is possible, it may have more subtle consequences on cell physiology. In particular, it may lead to a decrease in the rate of growth on some media and therefore secondary mutations may easily accumulate in cultures of cells which harbour the cloned gene. These mutations would lead to a decrease of, or alter the expression of, the cloned gene but may also affect the expression of several other genes in the harbouring cell.

The second conclusion is that this system seems to provide a means to select several classes of potentially useful mutants. It allows isolation of mutants located within the cloned gene. When these mutations correspond to an insertional inactivation of the gene, as found here, they can facilitate the mapping of the gene. Other types of mutations in the cloned gene might be expected which would not prevent the synthesis of the gene product, but only its export.

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