Molecular analysis of a *Clostridium butyricum* NCIMB 7423 gene encoding 4-α-glucanotransferase and characterization of the recombinant enzyme produced in *Escherichia coli*

Sayed K. Goda,1 Omima Eissa,2 Muhammad Akhtar2 and Nigel P. Minton1


1 Department of Molecular Microbiology, Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JE, UK

2 Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

An *Escherichia coli* clone was detected in a *Clostridium butyricum* NCIMB 7423 plasmid library capable of degrading soluble amylase. Deletion subcloning of its recombinant plasmid indicated that the gene(s) responsible for amylase degradation was localized on a 1.8 kb NsP1I–Seal fragment. This region was sequenced in its entirety and shown to encompass a large ORF capable of encoding a protein with a calculated molecular mass of 57 184 Da. Although the deduced amino acid sequence showed only weak similarity with known amylases, significant sequence identity was apparent with the 4-α-glucanotransferase enzymes of *Streptococcus pneumoniae* (46.9%), potato (42.9%) and *E. coli* (16.2%). The clostridial gene (designated *malQ*) was followed by a second ORF which, through its homology to the equivalent enzymes of *E. coli* and *S. pneumoniae*, was deduced to encode maltodextrin phosphorylase (*MalP*). The translation stop codon of *malQ* overlapped the translation start codon of the putative *malP* gene, suggesting that the two genes may be both transcriptionally and translationally coupled. 4-α-Glucanotransferase catalyses a disproportionation reaction in which single or multiple glucose units from oligosaccharides are transferred to the 4-hydroxyl group of acceptor sugars. Characterization of the recombinant *C. butyricum* enzyme demonstrated that glucose, maltose and maltotriose could act as acceptor, whereas the three only maltotriose could act as donor. The enzyme therefore shares properties with the *E. coli MalQ* protein, but differs significantly from the glucanotransferase of *Thermotoga maritima*, which is unable to use maltotriose as donor or glucose as acceptor. Physiologically, the concerted action of 4-α-glucanotransferase and maltodextrin phosphorylase provides *C. butyricum* with a mechanism of utilizing amylase/maltodextrins with little drain on cellular ATP reserves.

Keywords: MalQ, 4-α-glucanotransferase, *Clostridium butyricum*, starch degradation

**INTRODUCTION**

There is currently great interest in using biological fermentation as a possible alternative to fossil-fuel-based industries for the production of bulk chemical feedstocks and high-added-value chemicals. This is especially true of the genus *Clostridium*, members of which exhibit a diverse fermentative repertoire (Minton & Clarke, 1989). *Clostridium acetobutylicum* and its ability to produce the solvents acetone and butanol has attracted particular interest. Intensive research with this clostridial species has resulted in the development of gene transfer methodologies (Minton et al., 1993) and in the cloning of many of the genes encoding key enzymes of primary metabolism (Papoutsakis & Bennett, 1993).
Economic processes, such as the C. acetobutylicum-based acetone-butanol-ethanol (ABE) fermentation, will of necessity use low-cost, carbohydrate-based substrates such as amylose. The enzymes involved in amylose degradation in clostridia have, however, received comparatively little attention. This is despite the fact that amylose activity in C. acetobutylicum may be a limiting factor in solvent production (Lin & Blaschek, 1983; Hermann et al., 1985). Such a bottleneck could be circumvented by amplifying the endogenous levels of enzymes involved in amylose metabolism, or by endowing the organism with heterologous enzymes through gene cloning technology. With the latter strategy in mind, we constructed a gene library in Escherichia coli of the genome of an amylolytic strain of Clostridium butyricum, and screened for clones capable of degrading amylose. In the present communication we describe the characterization of one such clone, shown to produce a 4-a-glucosyltransferase analogous to the MalQ protein of the E. coli maltose regulon (Schwartz, 1987).

METHODS

Bacterial strains, plasmids and growth conditions. The source of chromosomal DNA was C. butyricum NCIMB 7423. The E. coli strains used as hosts for plasmid and M13 recombinants were strains TG1 [K-12, ∆(lac-proAB) supE thi hsdDS/F proA lacI2ZAM15] and E. coli CGSC#6153 [F- araD139 ∆(argF-lac) 205 fbl B5301 ptsF25 rpsL mtlQ63 bgI R15 deoC] respectively. Cloning vectors used were plasmids pMTL20/21 (Chambers et al., 1988) and pM55512 (Whelan et al., 1992), and phages M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). C. butyricum was cultivated in a semi-synthetic medium, essentially as described by Barker et al. (1959). E. coli was routinely cultivated in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). E. coli CGSC#6153 was cultivated in M9 minimal medium (Sambrook et al., 1989). Solidified medium consisted of L broth or M9 minimal media with the addition of 2% (w/v) agar (Bacto-Difco). The antibiotic used for the maintenance and the selection of transformants was 50 µg ampicillin ml⁻¹. Recombinant transformants were detected by their colourless appearance on L agar plates containing 100 µg IPTG ml⁻¹ and 50 µg X-Gal ml⁻¹.

DNA manipulations. Transformation of E. coli and large-scale plasmid isolation procedures were as previously described (Minton et al., 1983). Small-scale plasmid isolation was by the method of Holmes & Quigley (1981), whilst chromosomal DNA from C. butyricum was prepared essentially as described by Marmur (1961). Restriction endonucleases and DNA modifying enzymes were purchased from Northumbria Biologicals and were used under the conditions recommended by the supplier. Digests were electrophoresed in 1% (w/v) agarose slab gels on a standard horizontal system (Bio-Rad Model LH4), employing Tris/borate/EDTA (0.009 M Tris/borate, 0.002 M EDTA) buffer. The isolation of DNA restriction fragments from agarose gels using electroelution and DNA–DNA hybridizations were undertaken as described by Sambrook et al. (1989). Hybridizations were undertaken at 65 °C, followed by two 15 min washing periods in 6x SSC containing 0.1% (w/v) SDS at 55 °C. M13 template DNA was sequenced by the dideoxynucleotide method of Sanger et al. (1980) using a modified version of T7 polymerase, Sequenase (Tabor & Richardson, 1987). Analysis of generated nucleotide sequence data was undertaken using DNASTAR (Computer Genetics Group, Madison, WI, USA).

Identification and processing of recombinant clones. Recombinant clones capable of bringing about the breakdown of amylose were detected by screening for colourless halos around recombinant colonies on L agar supplemented with 1% (w/v) amylose following flooding with iodine solution (1%, w/v, iodine/potassium iodide). Colonies were lysed prior to staining by exposure to chloroform vapour. E. coli clones found to be producing an activity capable of degrading amylose were cultivated in 200 ml Luria broth supplemented with 30 µg ampicillin ml⁻¹ at 37 °C to an OD of 10 at 600 nm. Cells were harvested by centrifugation, washed in 20 ml 1× TE buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.0) and resuspended in 20 mM Tris/HCl, pH 7.5. Cell disruption was achieved by sonication (20 cycles s⁻¹, 2 A) for five 30 s intervals on ice. Cellular debris was removed by centrifugation at 17000 g for 40 min and the amylose-degrading activity precipitated with 45-55% saturated (NH)₄SO₄ and dialysed against water for 2 d prior to assay. Protein concentration was measured by the method of Bradford (1976), using BSA as the standard.

Enzyme assay. Glucanotransferase activity was quantified by measuring the reduction of amylose. The assay mixture contained 0.1% (w/v) maltose, 20 mM Tris/HCl, pH 7.5 and enzyme in a final volume of 3 ml. Following incubation at 37 °C, 0.1 ml samples were withdrawn at various time intervals, mixed with 1 ml 0.02% iodine/potassium iodide solution, and the decrease in absorbance at 620 nm measured. One unit of the enzyme activity was arbitrarily defined as the amount of enzyme which causes a change in absorbance of 1.0 in 15 min under the above conditions.

TLC chromatographic analysis. In certain instances the reaction products liberated were further characterized with respect to molecular size by thin-layer ascending chromatography on 0.2 mm silica-gel-coated aluminium sheets (type 60; Merck) employing a solvent system of 1 M lactic acid/acetone/2-propanol (2:4:3 by vol.). Carbohydrate spots were visualized by spraying the chromatogram with aniline/diphenylamine reagent (Sigma) and incubated at 160 °C for 10 min. In certain experiments use was made of [U-14C]maltose and [U-14C]glucose (Amersham). The radioactive products were visualized by autoradiography.

RESULTS

Isolation of recombinants able to degrade amylose

A gene library was constructed by inserting into the BamHI site of pMTL21 sized (4-7 kb) DNA fragments from C. butyricum NCIMB 7423 chromosomal DNA partially digested with Sau3A. Over 6000 putative recombinant clones were obtained and screened for the presence of amylose-degrading activity, using an in situ plate test. A total of four clones were identified which produced an enzyme activity capable of breaking down amylose within the vicinity of a lysed colony. Plasmid DNA was isolated from all four clones and subjected to digestion with a number of different restriction enzymes. This analysis indicated that no one clone contained a common DNA insert. The clone which appeared to be producing the most activity, as judged by halo size, was therefore selected for further analysis and its recombinant plasmid designated pSKG10. That this clone was
indeed capable of degrading amylose was confirmed by incubating crude cell lysates with amylose and measuring the observed change in intensity (ΔA₆₅₀) of iodine-staining. This indicated that cells of *E. coli* TG1 [pSKG10] grown for 16 h produced up to 20 U per mg total protein of amylose degrading activity per litre of culture.

**Characterization of the cloned gene**

Plasmid pSKG10 DNA was purified and shown to confer on a plasmid-free *E. coli* host the ability to degrade amylose when introduced by transformation. To confirm the clostridial origin of the cloned DNA, pSKG10 was radioactively labelled and used as a probe against chromosomal DNA isolated from *C. butyricum* NCIMB 7423 which had been digested with various restriction enzymes. In each case, pSKG10-derived DNA was shown to hybridize to discrete clostridial DNA fragments and not to control fragments derived from the *E. coli* chromosome (data not shown).

Having established the authenticity of pSKG10, a more detailed restriction map of its insert was derived (Fig. 1) and its size assessed as being 5.0 kb in length. To establish the position of the gene(s) responsible for amylose breakdown, a number of deletion sub-clones were made by excising subfragments from within the pSKG10 insert and re-cloning them into sites within the polylinker region of pMTL21. The ability of the resultant plasmids to elicit amylose degradation in *E. coli* was then tested using the *in situ* plate test. The regions sub-cloned are shown in Fig. 1. These experiments indicated that the smallest fragment which could be sub-cloned and still retain gene(s) responsible for amylose degradation was a 1.8 kb NspHI–Scal fragment (pSKG105, Fig. 1). Sub-cloning of a DNA fragment from which a further 425 bp had been removed from the NspHI end of this region resulted in a plasmid, pSKG104, which no longer conferred the ability to degrade amylose on *E. coli*. It was concluded that the gene(s) responsible resided between the NspHI and Scal sites.

**Nucleotide sequence determination**

To sequence the insert of pSKG105, some 50 templates carrying random 500 bp fragments were derived by the sonication procedure (Minton *et al.*, 1986). Prior to sonication, the isolated 1.8 kb NspHI (blunt-ended)–Scal fragment was circularized by self-ligation. The region analysed proved to be 1849 bp in length and to display an A+T content of 70 mol%, consistent with its
Fig. 2. Amino acid similarity between the 4-α-glucanotransferases of C. butyricum, S. pneumoniae and potato. Both identical and chemically similar amino acids are boxed. Identical amino acids are emboldened. C.BUT, MalQ of C. butyricum; S.PNE, MalM of S. pneumoniae; POTATO, 4-α-glucanotransferase of potato.
derivation from C. butyricum (Cummins & Johnson, 1973). Translation of the sequence revealed the presence of a single major ORF, initiating at position 118 with an AUG start codon and terminating at position 1581 with a UAA stop codon. The encoded polypeptide was therefore composed of some 487 amino acids and had a predicted molecular mass of 57 184 Da.

Comparative alignment of the deduced amino acid sequence of the polypeptide encoded by the identified ORF with characterized amylase or glucoamylase sequences indicated only a low level of similarity. Significant homology (46.9% identity) was, however, apparent (Fig. 2) with the published sequence of the MalM protein of Streptococcus pneumoniae (Lacks et al., 1982) and (42.9% identity) with α-glucanotransferase of potato (Takahashi et al., 1993). These proteins correspond to α-glucanotransferase, or formerly amylomaltase (Palmer et al., 1976). In contrast, little homology (16.2% identity) was evident between the clostridial protein and the extensively characterized 4-α-glucanotransferase of E. coli (Pugsley & Dubreuil, 1988). A similar lack of similarity between the S. pneumoniae MalM protein and α-glucanotransferase of potato (Takahashi et al., 1993) with the E. coli MalQ enzyme has been previously noted (Pugsley & Dubreuil, 1988). Despite this low level of similarity, the enzymic properties of the clostridial protein (see below) are identical to those reported for the E. coli malQ gene product. In view of this functional identity, the clostridial enzyme was designated MalQ, and its encoding gene malQ.

The enzyme 4-α-glucanotransferase (EC 2.4.1.25) is involved in maltose and maltooligosaccharide metabolism. In both E. coli and S. pneumoniae these enzymes act in concert with a phosphorylase (EC 2.4.1.1), the encoding gene of which is located within the immediate vicinity of both malQ and malM. An examination of the nucleotide sequence of the NspH–ScaI fragment revealed the large ORF was followed after its stop codon by a incomplete ORF, which apparently terminated at a UAA stop codon. The encoded polypeptide was therefore composed of some 487 amino acids and had a predicted molecular mass of 57 184 Da.

**Characterization of the recombinant enzyme**

The ability of E. coli-derived recombinant cell-free extracts to bring about the release of glucose from amylose had been demonstrated when the gene was initially isolated through the use of a glucose oxidase assay (data not shown). However, other enzymes besides glucotransferases liberate glucose from amylose, most notably glucoamylase or α-amylase. To definitely establish that the C. butyricum malQ gene did indeed encode a glucanotransferase, a further series of experiments were undertaken.

In the first instance, plasmid pSKG105 was shown to complement the malQ mutation of the E. coli strain CGSC#6153 by plating transformed cells on M9 minimal agar containing maltose as sole carbon source. Transformant colonies were not obtained in control experiments in which the plasmid utilized was the cloning vector pMTL21. Using CGSC#6153 transformed with pSKG105, further evidence that the clostridial MalQ protein is a glucanotransferase was obtained when it was demonstrated that the size of the zone of amylose degradation around a lysed recombinant colony was significantly increased by the presence of maltose (1%) in the agar medium (data not shown). This stimulatory effect could be measured more directly by monitoring the change in iodine-staining properties during the conversion of amylose, or a mixture of amylose and maltose, over a period of time. The data obtained (Fig. 3) demonstrated that the presence of maltose in the reaction mixture abolished the considerable lag in amylose breakdown observed when cell extracts were incubated in the presence of amylose alone. This lag in amylose degradation may be attributable to the initial absence of a suitable acceptor, such as maltose, for amylose-derived glucansyl units. A delay in enzyme activity in the absence of maltose would not occur if the clostridial enzyme was an amylace.

Glucanotransferase catalyses a disproportionation reaction in which single or multiple glucose units from oligosaccharides are transferred to the 4-hydroxyl group of acceptor sugars. It follows that in the presence of a glucanotransferase and amylose, radioactive label ini-
The presence of amylose (a) or maltotriose (b). Reactions were performed at 37°C in 1 ml 20 mM Tris/HCl, pH 7.5 and contained 0.01 ml of a cell-free extract derived from E. coli CG5SC#6153 cells containing pSKG105, 100 μg soluble amylose (a) or 100 μg maltotriose (b), and 1 μCi (37 kBq) [14C]maltose. At various time intervals, 100 μl samples were withdrawn, and subjected to TLC. In (a), the samples in lanes 6–11 were withdrawn after 0, 5, 10, 15, 20 min and 24 h, respectively. Lanes 1–4 represent the corresponding control experiment (samples withdrawn after 0, 5, 10 and 20 min, respectively, where extracts were derived from cells carrying the cloning vector alone). In (b), the reaction was carried out as described in (a) but maltotriose was used in place of amylose. The samples in lanes 9–15 were withdrawn after 0, 5, 10, 15, 20, 25 and 30 min, respectively. Lanes 1–7 represent the corresponding control. The positions of the molecular mass standards used in the assay are marked on the right of each chromatogram: G1, glucose; G2, maltose; G3, maltotriose; G6, maltohexaose; and G7, maltoheptaose. One of the labelled species being maltotriose. This demonstrates that the clostridial enzyme can use maltose as an acceptor to which it is able to transfer a glucosyl (i.e. single glucose) unit to yield maltotriose. The formation of higher 14C-containing sugars emphasizes that the enzyme may also transfer glucanosyl units (oligomers of glucose). Essentially identical results were obtained when amylose was incubated with [14C]glucose (data not shown), demonstrating that this sugar could also act as an acceptor molecule. In all cases, whatever the acceptor, it was evident that after 24 h incubation, glucose and maltose were the sole final reaction products (Fig. 4a). This underlines the fact that, as is the case with the E. coli MalQ enzyme, glucose and maltose act only as acceptors and not donors of glucose units. As shown by Palmer et al. (1976), the production of glucose from maltose by such enzymes depends on the presence of catalytic amounts of higher oligodextrins. These act as glycosyl donors, which are continuously recycled upon transfer of the glycosyl moiety to a maltose acceptor.

Further light on the substrate specificity of the enzyme was shed by the profile of products obtained from the incubation with [U-14C]maltose and unlabelled maltotriose. The formation of labelled maltotriose in such experiments (Fig. 4b) once again emphasized that a glucosyl unit from maltotriose may be transferred to maltose. This observation, together with the labelling of higher sugars, demonstrated that maltotriose can act as a donor sugar.

**DISCUSSION**

In the present study we have used an in situ plate test to identify a clostridial gene, malQ, encoding 4-α-glucanotransferase. In common with the equivalent genes of E. coli and S. pneumoniae, the gene encoding a malto-dextrin phosphorylase is located in the immediate vicinity of the 4-α-glucanotransferase gene. In E. coli, the two genes form an operon, in which the malP gene precedes malQ. In both C. butyricum NCIMB 7423 and S. pneumoniae the order is reversed. Whether the two C. butyricum genes are co-transcribed was not established. However, their extremely close proximity suggests that this is extremely likely. Furthermore, as the stop codon of the clostridial malQ overlaps the start codon of malP (Fig. 1), there is a strong possibility that the two genes are translationally coupled. The evolution of active measures to ensure the co-ordinate production of the two encoded enzymes is consistent with their important complementary roles in maltdextrin metabolism.

On the smallest DNA fragment subcloned, the 1.8 kb NspHI–Scal fragment, the malQ gene is preceded by only 117 bp. The identity of sequences within this region important in the expression of malQP was not determined. A sequence typical of clostridial ribosome-binding sites (Young et al., 1989) is present immediately 5’ to the malQ start codon, but the identification of sequences important in transcription will require further experimental analysis. Direct evidence that transcription signals are present was obtained when this fragment was...
inserted into the polylinker region of plasmid pMTL32 to give pSKG132. Cells carrying pSKG132 were shown to produce equivalent levels of amylase-degrading activity to the same E. coli cells carrying plasmid pSKG105. Thus, as the cloning sites of pMTL32 are flanked by transcriptional terminators, transcription of malQ in pSKG132 must initiate from an endogenous promoter. As these experiments were undertaken in E. coli, it remains to be seen whether this same region has promoter activity in C. butyricum.

The identity of the clostridial MalQ protein as a glucanotransferase, rather than an amylase or glucosamylase, was indicated by the demonstration that single or multiple glucose units were transferred from oligosaccharides to the acceptor sugars, glucose, maltose or maltotriose. The occurrence of multiple disproportionation reactions during in vitro incubation meant that it was not possible to determine the size of the largest glucanosyl units which can be transferred. However, the formation of products of ten glucose units in incubations performed with amylase and maltose (Fig. 4b) could indicate that at least eight glucanosyl units may be transferred by the clostridial enzyme. Following an extended period of incubation (lane 15, Fig. 4b), radioactivity was solely distributed amongst maltose and glucose. Whilst the generation of oligodextrins from maltose shows that the C. butyricum MalQ is a glucanotransferase, the degradation of amylase, as measured by reduced binding of iodine, suggests that some hydrolytic activity may provide low-molecular-mass acceptors. Alternatively, low-molecular-mass glucosyl acceptors may be generated by MalQ.

Our studies also established that both glucose and maltose, in addition to maltotriose, could act as acceptor molecules. The action of the enzyme on maltotriose in the presence of excess [14C]glucose and the formation of radioactive maltotriose (data not shown) indicates that maltotriose could also act as a donor. In this respect the properties of the clostridial 4-glucanotransferase are similar to those for the corresponding enzyme (MalQ) from E. coli (Pugsley & Dubreuil, 1988) and α-glucanotransferase of potato (Takahashi et al., 1995). They differ from those of the enzyme (GTase) isolated from Thermotoga maritima (Liebl et al., 1992), which uses neither maltotriose as the donor sugar nor glucose as the acceptor sugar.

Physiologically, the concerted action of α-glucanotransferase and maltodextrin phosphorylase provides the cell with a mechanism of utilizing maltodextrins with little drain on cellular ATP. Assuming that the MalP enzyme exhibits similar catalytic properties to the equivalent E. coli protein, the co-operative action of the clostridial MalQ and MalP enzymes may be illustrated by a hypothetical example involving maltotriose. After three rounds of disproportionation, 4-glucanotransferase may produce maltodextrin containing up to nine glucose units (i.e. maltonaose) and release three equivalents of glucose. For the further metabolism of glucose, ATP is required. However, maltonaose will enter the hexose phosphate pool, by the action of maltodextrin phosphorylase, until maltopentaose is produced which, as maltodextrin phosphorylase is inactive against oligosaccharides of less than six sugar units in length, is no longer a substrate for the enzyme. Maltopentaose, through the action of 4-glucanotransferase, may undergo further rounds of disproportionation producing higher maltodextrins for use by maltodextrin phosphorylase. In addition to their catabolic role, the two enzymes may also be involved in the storage process by transferring the maltodextrin skeleton to acceptors producing higher polymers. The role of 4-α-glucanotransferase in the biosynthesis of long 1,4-glucan (amylose) is shown by the properties of E. coli mutants devoid of maltodextrin phosphorylase (Schwartz, 1987). These mutants, when grown in the presence of maltodextrin, accumulate large amounts of amylase, formed through a succession of disproportionation events catalysed by the transferase.

Concluding remarks

In the present study we have isolated a gene from clostridia which encodes a product capable of degrading starch. On the basis of the following observations, the encoded enzyme was concluded to encode an α-4-glucanotransferase: the enzyme catalysed the synthesis of higher molecular mass oligosaccharides; activity was stimulated by the presence of maltose; the gene was able to complement an E. coli malQ mutant; and the deduced amino acid sequence exhibited significant identity with characterized α-4-glucanotransferase enzymes, including those of S. pneumoniae (46.9%), and potato (42.9%).

ACKNOWLEDGEMENTS

The authors wish to thank Nicola Minion for typing this manuscript. N.P.M wishes to acknowledge the financial support of the BBSRC, research grant T04089.

REFERENCES


Received 20 March 1997; revised 5 June 1997; accepted 10 June 1997.