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Cloning and phenotypic expression in *Escherichia coli* of a *Bacillus subtilis* gene fragment coding for sucrose hydrolysis

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Summary

A DNA fragment from *Bacillus subtilis* strain Marburg coding for the synthesis of an enzyme catalyzing sucrose hydrolysis was cloned in *Escherichia coli* and detected using simple direct selection of transformants growing on sucrose. Three different clones were obtained each having a 2.5 kb *EcoRI-PstI* fragment in common which was shown to be sufficient to mediate growth on sucrose. This fragment was not identical with known cloned gene fragments of *B. subtilis* coding for the sucrose hydrolyzing enzymes sucrase and levansucrase. It could be shown that the 2.5 kb fragment codes for a third sucrose hydrolyzing enzyme, namely for levanase. In the case of *E. coli* this enzyme was found to be mainly intracellular; however, a small quantity was also excreted into the periplasmic space.

recombinant DNA, sucrose hydrolysis, inulin, levanase, *Bacillus subtilis*, *Escherichia coli*

Introduction

The use of carbohydrates as renewable sources for the production of chemicals, food and energy is subject to increasing interest (Dunnill, 1981; Righelato, 1980). Thus enzymes involved in carbohydrate metabolism are of great importance both

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for the biotechnological and food industries. The production of such enzymes is one particular reason to clone genes coding for those specific proteins. Another particular aspect is to have available those genes for the purposes of strain improvement using recombinant DNA techniques with microorganisms already employed in bioprocesses (Schwab, 1981).

The genus *Bacillus* is a source of many different hydrolytic enzymes which are mostly excreted from the cell (Priest, 1977). In the case of *Bacillus subtilis*, three enzymes capable of hydrolyzing sucrose have been described (Lepesant et al., 1972; Lepesant et al., 1974; Kunst et al., 1977). The corresponding genes for two of these enzymes have been recently cloned in *E. coli*. However, phenotypic expression in *E. coli* could not be detected either for sucrase (Fouet et al., 1982) or for levansucrase (Gay et al., 1983). On the other hand, intracellular production of both of these enzymes in *E. coli* has been reported by the authors cited above.

The results presented here pertain to the cloning and characterization of a third, different gene from *B. subtilis* which codes for sucrose hydrolysis and, furthermore, enables strains of *E. coli* containing this fragment to grow on sucrose as the sole carbon source.

Materials and Methods

Bacterial strains, cloning vectors, media and culture conditions

Bacillus subtilis strain Marburg was obtained from the "Deutsche Sammlung von Mikroorganismen" (DSM 10). *Escherichia coli* C600r⁻ was used as a host for recombinant plasmids and was obtained from W. Wenzel, Cold Spring Harbour Laboratories. The *E. coli* strain DS410 producing minicells was a gift from A. Pühler, University of Bielefeld, F.R.G. The vector plasmid pBR322 (Bolivar et al., 1977) was provided by P. Starlinger, University of Cologne, F.R.G.

Bacterial cells were routinely grown at 30 or 37°C in nutrient broth (8 g l⁻¹, Difco instant preparation) or in LB medium (10 g l⁻¹ bacto tryptone, 5 g l⁻¹ bacto yeast extract, 5 g l⁻¹ NaCl). For the selection of plasmids the antibiotics tetracycline (15 mg l⁻¹) or ampicillin (100 mg l⁻¹) were added as required. To select for growth on sucrose a M9 mineral salts medium (Miller, 1972) supplemented with essential amino acids (20 mg l⁻¹) and with thiamine (1 mg l⁻¹) and also containing sucrose (2 g l⁻¹) as the sole carbon source was used. Sucrose containing no other monosaccharides (Sigma) was separately sterilized by filtration (0.2 µm membrane filters) to avoid hydrolysis by heat sterilization. Media were usually solidified by the addition of 1.5% agar.

Cloning procedures and restriction analysis

Chromosomal DNA from *B. subtilis* was extracted according to the method of Saito and Miura (1963). Minipreparations of plasmid DNA from *E. coli* strains for rapid analysis of clones were obtained by the alkaline lysis method of Birnboim and Doly (1979) with slight modifications. Preparation of purified plasmid DNA used for cloning purposes and for detailed restriction analysis was done by CsCl/ethidium

bromide (EtBr) gradient centrifugation of cell lysates prepared by alkaline lysis of cells as described in Maniatis et al. (1982). After removal of EtBr by extraction with water-saturated *n*-butanol the DNA was dialyzed against TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and concentrated by ethanol precipitation. DNA preparations were stored in TE buffer at 4°C or at -20°C.

All cloning procedures were performed according to standard methods as described in Maniatis et al. (1982). Restriction endonucleases and DNA modifying enzymes were purchased either from BRL (F.R.G.) or from Boehringer Mannheim (F.R.G.) and used under conditions as recommended by the suppliers. Agarose gels were usually run in the sub-cell mode. For the separation of small DNA fragments vertical polyacrylamide slab gels were used. In each case a Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.0) was used. Elution of DNA fragments from agarose gels was performed by the freeze-squeeze method as described by Thuring et al. (1975).

Determination of enzyme activities

For the determination of inulin degrading activities according to Kunst et al. (1977), cells were cultured in 300 ml sucrose mineral salts medium for 48 h at 30°C. The cells were harvested by centrifugation, washed with 0.9% NaCl and finally suspended in 10 ml citrate buffer (0.32 M, pH 4.6). The cells were then disrupted with a French press (800 lb/in², 0°C). 1 ml of the cell lysate was mixed with 1 ml of reaction buffer (0.2 M Na₂HPO₄, 0.2 M sodium acetate, 10 mM inulin, pH 5.0) and incubated at 37°C. Samples of 0.1 ml were taken at regular intervals and heated for 5 min at 95°C to inactivate all enzymes. After centrifugation to remove all cellular particles, the fructose content in the supernatant was determined enzymatically (Boehringer Mannheim, F.R.G.).

Saccharolytic enzyme activities with respect to their intracellular, periplasmic or extracellular location in *E. coli* cells were determined as follows: Cells from a 48-h culture on sucrose mineral salts medium (50 ml) were harvested by centrifugation; the supernatant was designated the extracellular fraction (fraction a). Cells from the same culture were washed with 0.9% NaCl, suspended in 5 ml citrate buffer (0.32 M, pH 4.6) and disrupted with a French press as described. This French press lysate represents the total of intracellular and periplasmic enzymatic activities (fraction b). Fractions solely representing periplasmic enzymatic activity were prepared according to the method described by Cornelis et al. (1982). With this procedure enzymes are released from the cells by gentle osmotic shock treatment (fraction c).

All of these fractions were examined for their ability to catalyse sucrose hydrolysis. Experimentally a buffered sucrose solution (0.5% sucrose in 0.32 M citrate buffer, pH 4.6) was added to each fraction in ratios of 1:1 to 1:10 and the mixtures were then incubated at 37°C. Samples were removed at regular time intervals, heated to 95°C for 5 min and clarified by centrifugation. The glucose content of the supernatant was determined enzymatically using a commercial test combination (Boehringer Mannheim, F.R.G.).

Analysis of gene products with minicells

Purified DNA from plasmids to be analysed was transformed into the minicell

producing strain *E. coli* DS410. Plasmid DNA from the proper phenotypical transformants was subjected to restriction analysis in order to check for the correct plasmid genotype. Cells of the analysed transformants were grown in 250 ml LB medium containing tetracycline (10 mg l^{-1}) to an optical density of 4–5 (420 nm, $d = 1.0 \text{ cm}$). Viable cells were removed by low speed centrifugation ($600 \times g$, 10 min). The supernatant containing the minicells was then centrifuged at $10\,000 \times g$ for 15 min to harvest the remaining cells. Minicells were then purified by four sequential centrifugations through 10–30% sucrose gradients as described by Clark-Curtiss and Curtiss (1983). Labeling of proteins produced by the minicells was performed with [^{35}S]methionine according to Meagher et al. (1977). After lysis by SDS, the proteins were separated on vertical polyacrylamide-SDS gels and subsequently detected by autoradiography using X-ray film (Kodak type S3).

Results

Cloning of Bacillus subtilis DNA fragments coding for sucrose hydrolysis

Chromosomal DNA of *B. subtilis* strain Marburg was partially digested with the restriction endonucleases *EcoRI* and *PstI*. Conditions for cleavage resulting in a maximum of fragments within a size range of 2–5 kb were determined in preceding experiments by variation of enzyme concentration and duration of incubation. Finally, 10 μg of *B. subtilis* DNA were simultaneously digested with 5 U *EcoRI* and 5 U *PstI* in a 100 μl reaction mixture for 60 min at 37°C .

DNA of the plasmid pBR322 purified by density gradient centrifugation was digested with the same enzymes and the resulting fragments were separated on a 0.7% agarose gel. The large 3.6 kb fragment of pBR322 was eluted from the gel by the freeze-squeeze method (Thuring et al., 1975). *B. subtilis* DNA fragments generated by partial *EcoRI* and *PstI* digestion were ligated to the large *EcoRI-PstI* fragment of pBR322 using T4 DNA ligase and transformed into *E. coli* C600 r^- . Tetracycline-resistant, ampicillin-sensitive transformants were selected and subsequently screened on M9-sucrose agar plates. From more than 1000 clones tested, three clones were isolated that were capable of growing on sucrose. Although the growth of these clones on sucrose mineral medium was much slower than on glucose media, colonies growing on sucrose media could be clearly identified after 2 days incubation. In the case of these three isolated clones no significant difference could be detected with respect to growth behaviour.

Restriction analysis of hybrid plasmids

Plasmid DNA was isolated from each of the three sucrose-positive *E. coli* clones and analysed with the restriction enzymes *EcoRI* and *PstI*. It was found that all hybrids contain a 2.5 kb fragment in addition to the 3.6 kb vector fragment. The plasmid pKF3 consists solely of the 3.6 kb vector fragment and the 2.5 kb *B. subtilis* DNA fragment. In contrast to the other two hybrids pKF1 and pKF2 it does not contain additional DNA (Fig. 1). Therefore, it is highly probable that this 2.5 kb fragment contains the genetic information for sucrose hydrolysis. In order to

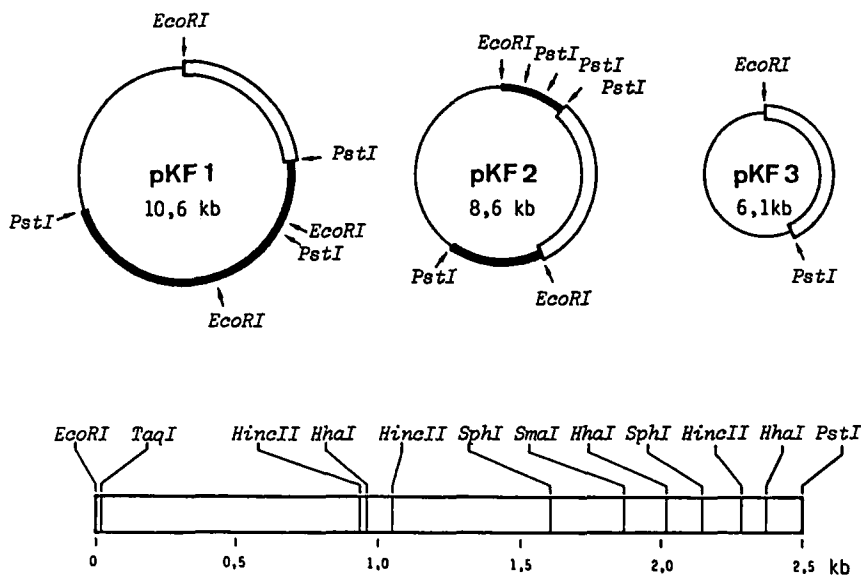


Fig. 1. Restriction maps of the recombinant plasmids mediating sucrose hydrolysis. The fine line represents the vector part (*EcoRI/PstI* fragment of pBR322). The heavy line represents *B. subtilis* DNA not essential for sucrose hydrolysis. The double lined segment represents the 2.5 kb *EcoRI/PstI* *B. subtilis* DNA fragment common to all three plasmids and encoding sucrose hydrolysis. A more detailed map of this fragment is shown in the lower part of the drawing.

investigate this fact, the 2.5 kb fragments from pKF1 and pKF2 were subcloned into the 3.6 kb *EcoRI-PstI* fragment of pBR322 and transformed into *E. coli* C600r⁻. Hybrids containing solely the 2.5 kb fragment could be isolated from both sources, i.e. from pKF 1 and pKF2 respectively. All such clones tested in more detail (8 each) showed growth on sucrose when supplied as the sole carbon source.

By digestion of DNA of the plasmids pKF1, pKF2 and pKF3 with further restriction enzymes, it was possible to physically confirm that the 2.5 kb fragment is identical in all of the three different clones. It could also be determined that the 2.5 kb fragments are located in different orientations with respect to the vector parts in the plasmids pKF1 and pKF3 in comparison to pKF2 (Fig. 1).

The 2.5 kb fragment was further analysed in more detail by means of restriction enzyme digestions of the plasmid pKF3 and of purified DNA of the 2.5 kb *EcoRI-PstI* fragment obtained by freeze-squeeze elution from agarose gels. A map of this fragment is shown in Fig. 1. In addition to the marked positions for restriction sites of several enzymes, it was found that there are no cleavage sites located on the 2.5 kb fragment for the restriction endonucleases *Bam*HI, *Bgl*II, *Hae*II, *Hind*III, *Kpn*I, *Sal*I, *Sst*II and *Xho*I.

Characterization and localization of enzyme activities in E. coli

By comparing our results of the restriction enzyme analysis with the published

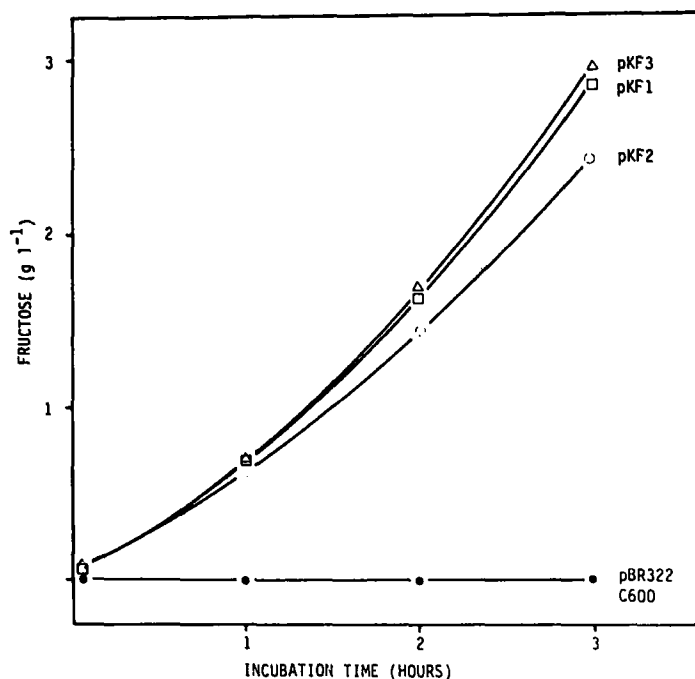


Fig. 2. Inulin degradation abilities of cell lysates of *E. coli* C600^r containing recombinant plasmids as measured by the formation of free fructose from inulin. The respective plasmids are marked at the right end of the corresponding curves. The plasmid-free host strain, marked as "C600", and the strain containing the vector plasmid pBR322 without any *B. subtilis* DNA insert were also analyzed for comparison.

data pertaining to cloned gene fragments of *B. subtilis* containing the sucrase (Fouet et al., 1982) and the levansucrase gene respectively (Gay et al., 1983), we found that there are no existing common features between these fragments and the 2.5 kb fragment. Therefore, it was obvious to look for indications of the third possible sucrose hydrolyzing enzyme in *B. subtilis*, namely levanase (Kunst et al., 1977). Levanase is the only one of the three enzymes which is capable of hydrolyzing the fructosan inulin, a molecule consisting of approximately 30 β -1,2-connected fructose units with a sucrose molecule at one end.

Whole cell lysates from *E. coli* clones harbouring plasmids pKF1, pKF2 or pKF3 were tested for inulin hydrolyzing activities as described in Materials and Methods. As can be seen from Fig. 2, all lysates prepared from the three different clones show nearly identical inulin degrading activities. Therefore, it may be concluded that the 2.5 kb fragment carries the genetic information for the enzyme levanase.

No growth of *E. coli* strains containing either the cloned sucrase gene or the cloned levansucrase gene (Fouet et al., 1982; Gay et al., 1983) could be detected even though the corresponding enzymes were produced intracellularly. Thus it was very interesting to determine just where the levanase activity is located in *E. coli*.

Extracellular, periplasmic and intracellular enzyme activities were therefore determined. It was found that no extracellular sucrose hydrolyzing activity is present in culture fluids (fraction a) following growth of *E. coli* strains harbouring pKF1, pKF2 or pKF3 plasmids. However, a low activity could be detected in fractions representing periplasmic enzymes (fraction c). But in comparison to fractions b which represent intracellular plus periplasmic activities, and where the major activity could be found, the periplasmic activity represents only about 0.2–1% of the total activity. On the basis of these data we suggest that the levanase is at least to a small extent transported into the periplasmic space of *E. coli* cells thus resulting in the ability to grow on sucrose media.

Identification of plasmid encoded proteins in minicells

A series of minicell experiments were performed with pKF plasmids in order to detect proteins responsible for sucrose hydrolysis and much effort was put into minicell purification. The quality of the minicell preparations was found to be quite satisfactory with respect to vector encoded proteins, however, no visible protein bands specific for the 2.5 kb fragment could be detected.

Since the synthesis of proteins is limited by the amount of RNA polymerase present in minicells, strong promoters bind most of the available RNA polymerase resulting in a very low expression of genes transcribed from weak promoters (Crooks et al., 1983). Therefore, as an explanation for the fact that proteins encoded by the 2.5 kb fragment could not be clearly detected, we assume that such proteins are transcribed in *E. coli* from very weak promoters possibly being those from *B. subtilis*.

Discussion

A 2.5 kb *EcoRI-PstI* fragment of the chromosomal DNA of *Bacillus subtilis* strain Marburg, cloned into the 3.6 kb *EcoRI-PstI* fragment of pBR322, was shown to code for sucrose hydrolyzing activity. In contrast to recently described cloned *B. subtilis* DNA fragments coding for the enzymes sucrose (Fouet et al., 1982) and levansucrose (Gay et al., 1983), this fragment enables cells of *E. coli* to grow on sucrose as the sole carbon source. Since the identical 2.5 kb fragment was found to be present in three different hybrid plasmids it is suggested that this fragment codes for a specific sucrose hydrolyzing enzyme.

No significant difference with respect to the growth behaviour on sucrose between the *E. coli* clones containing the different plasmids pKF1, pKF2 or pKF3 could be found. The plasmid pKF3 contains only the 2.5 kb *B. subtilis* DNA fragment whereas pKF1 and pKF2 contain this fragment and additional *B. subtilis* DNA. Furthermore, the 2.5 kb fragment is located in a different orientation in one of the hybrids compared to the other two (Fig. 1). Thus these facts might be good evidence that expression of the sucrose hydrolyzing enzyme in *E. coli* will be controlled by a *B. subtilis* promoter located within the 2.5 kb fragment. In addition, *E. coli* C600 clones containing a hybrid plasmid consisting of the 2.5 kb *B. subtilis*

DNA fragment cloned into the broad host range vector pMMB33 (Frey, 1983) were found to grow on sucrose with similar characteristics as found with *E. coli* C600 clones containing one of the pKF plasmids (Friebs, unpublished results). An interesting fact was the finding of a sucrose hydrolyzing activity in the periplasmic space of *E. coli* clones harbouring pKF plasmids, although the main enzyme activity was located intracellularly. Nevertheless, this fact may give rise to the supposition that the *B. subtilis* secretion information may at least be poorly expressed in *E. coli*. However, additional data are necessary to allow more precise conclusions.

As could be found by detailed restriction analysis of the 2.5 kb *B. subtilis* DNA fragment, no homology is given with respect to the cloned gene fragments coding for sucrase (Fouet et al., 1982) or levansucrase (Gay et al., 1983). By testing cell lysates of *E. coli* clones harbouring plasmids containing the 2.5 kb fragment for inulin hydrolyzing activities, much evidence was obtained for the fact that this fragment codes for the enzyme levanase. This enzyme is the only one of the sucrose hydrolyzing enzymes in *B. subtilis* which is able to hydrolyse inulin. However, studies of the growth behaviour of *E. coli* strains harbouring pKF plasmids on mineral salts media containing inulin as the sole carbon source showed that growth on inulin was much slower compared to growth on sucrose. We suggest that sucrose or inulin must be transported across the outer membrane of *E. coli* to the periplasmic space and is then hydrolyzed to monosaccharides which can be taken up into the cell. Apparently such a sequence would cause more difficulties to the utilization of the larger inulin molecule as a carbon source.

In looking for proteins coded for by the 2.5 kb fragment by analysing newly synthesized proteins produced by minicells harbouring the plasmid pKF3, we were not able to detect protein bands clearly specific for the 2.5 kb fragment even though a large number of experiments using highly purified minicell preparations were performed.

One suggestion is that the expression of the levanase is controlled by a promoter which shows only a weak activity in *E. coli* and therefore, in minicells the quantity of protein synthesized is too low to be clearly detected. This would support the idea that expression of the sucrose hydrolyzing enzyme (levanase) which is encoded by the 2.5 kb *B. subtilis* DNA fragment is controlled by a *B. subtilis* promoter which shows only weak activity in *E. coli*.

A more detailed analysis of the 2.5 kb fragment by sequencing should provide better information concerning encoded genes and their expression. Experiments for this purpose have been already started.

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