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Cloning and expression of the levanase gene in *Alcaligenes eutrophus* H16 enables the strain to hydrolyze sucrose

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Summary

Genetic engineering methods were used to enhance the substrate spectrum of *Alcaligenes eutrophus* H16, a poly-β-hydroxybutyric acid (PHB) producer. Using parts of the vector pMMB33 and a 2.5 kb DNA fragment of the *Bacillus subtilis* chromosome a plasmid was constructed bearing the gene for levanase, an enzyme able to hydrolyze various saccharides. After transfer of the levanase gene by triparental conjugation, the gene, controlled by its own *Bacillus subtilis* promoter, is expressed in *Alcaligenes eutrophus* H16 and enables the strain to hydrolyze sucrose. However, growth on sucrose is limited; i.e. the sucrose is not transported efficiently into the cell and/or the levanase is not secreted into the medium.

Alcaligenes eutrophus; Recombinant DNA; Sucrose hydrolysis; Levanase

Introduction

Alcaligenes eutrophus H16, a chemolithoautotrophic bacteria, produces the biopolymer poly-β-hydroxybutyric acid (PHB). This biopolymer has thermoplastic properties similar to those of polypropylene and is biodegradable, unlike synthetic plastics (King, 1982; Lafferty and Heinzle, 1977). The advantage of environmental

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protection and the possibility to use renewable resources as substrates, give PHB a large commercial potential. The British company Marlborough Biopolymers Ltd. produces PHB by cultivation of *Alcaligenes eutrophus* and is investigating various applications. Besides the possibility of using PHB as a raw material for producing everyday articles like bottles, PHB can be used in medicine. Investigations have been performed to study its use in the formation of long-term release drugs, medical one-way articles, or for bone repair (Lafferty et al., 1988).

The likelihood of PHB production on an industrial scale and its wide commercial application depends on the competition with the costs of producing synthetic polymers. *Alcaligenes eutrophus* H16, which can produce PHB in amounts up to 90% of its total dry weight cannot be cultivated using substrates such as sucrose-containing byproducts. The wild type can only use fructose out of all sugars and some mutants are also able to use glucose too. One step towards improving the economics of PHB production with *A. eutrophus* would be to enlarge the substrate spectrum for this bacteria.

Using genetic engineering methods, it should be possible to supply *A. eutrophus* with genes enabling it to use cheaper raw materials or even perhaps waste products. Genes carrying the information for enzymes, able to degrade various types of carbohydrates are of interest for transfer into *A. eutrophus* H16. Such enzymes include all types of sucrases, amylases, cellulases, and other hydrolases. *Bacillus subtilis* produces three exoenzymes with sucrose-hydrolyzing properties (Lepesant et al., 1972, 1974; Kunst et al., 1977). In addition to sucrose one of these enzymes, levanase, is capable of hydrolyzing several polysugars such as inulin, a polyfructan. The gene for this levanase has been cloned in *E. coli* and is available on a plasmid (Friehs et al., 1986). This report describes the transfer and expression of levanase in *A. eutrophus* H16, which allows this strain to utilize sucrose-containing media.

Materials and Methods

Bacterial strains, cloning vectors, media and culture conditions

E. coli C600 (ATCC 23724) and *A. eutrophus* H16 (ATCC 17699) were used as hosts for recombinant plasmids. The vector pMMB33 (Frey, 1983) and the strain *E. coli* J53, carrying the helper plasmid RP4-4, were gifts from Prof. Pühler (Pühler, 1983). The plasmid pKF3, carrying the genes for the levanase located on a 2.5 kb fragment of *B. subtilis* DNA, has been described previously (Friehs et al., 1986). The vector pKF4, a hybrid of a fragment of pMMB33 and the 2.5 kb fragment of *B. subtilis* DNA on the plasmid pKF3, was constructed in this work.

All bacteria were routinely grown under aerobic conditions at appropriate temperatures either in 'nutrient broth' (NB, 8 g l^{-1} Difco instant preparation) or LB media (10 g l^{-1} casamino acids, 5 g l^{-1} yeast extract, and 10 g l^{-1} NaCl). For solid media, 1.5% (w/v) agar was added. Two media were used for testing growth on sucrose as a carbon source: for *E. coli*, the M9 mineral salts medium decribed by Miller (Miller, 1972), and for *A. eutrophus* H16, the mineral salts media of Schlegel et al. (1961), each containing 2% sucrose. Other media used are described in the text.

Isolation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method, as described by Schwab et al. (1983). Purification of plasmid DNA was done by CsCl-ethidium bromide density-gradient centrifugation. For rapid analysis by restriction enzyme digestion, mini-preparations were performed, as described by Maniatis et al. (1982) and Ish-Horowicz and Burke (1981).

DNA manipulations

Digestion of plasmid DNA with restriction enzymes was done under the conditions recommended by the suppliers (BRL, F.R.G. or Boehringer Mannheim, F.R.G.). Agarose gels were run in the sub-cell mode using a Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.0). Elution of DNA fragments from the agarose gels was performed by the 'freeze-squeeze' method as described by Thuring et al. (1975). All cloning procedures were performed according to standard methods as described in Maniatis et al. (1982).

Plasmid transfer and screening

Transformation of *E. coli* C600 was done by growing it in NB medium at 37 °C, preparing competent cells and transforming them using the calcium chloride procedure described by Maniatis et al. (1982). Conjugative plasmid transfer into *A. eutrophus* was performed by triparental mating, mixing the donor, the recipient, and the strain carrying the helper plasmid on solid NB medium. After 48 h of incubation at 30 °C the mixture was plated on different solid media to screen for *A. eutrophus* H16 clones carrying pKF4.

Determination of levanase activity

The inulin-degrading activities of the pKF4-harboring strains were determined by the method of Kunst et al. (1977). Cells were grown in 250 ml LB medium containing sucrose and 200 mg l^{-1} kanamycin, harvested by centrifugation, washed with citrate buffer (0.32 M, pH 4.6), resuspended in 10 ml of the same buffer and frozen. One ml of thawed cell suspension was used in the assay. The cells were disrupted by sonication (100 W for 3 min, in an ice bath). Lysate (0.5 ml) was mixed with 0.5 ml of reaction buffer (0.2 M Na_2HPO_4, 0.2 M sodium acetate, 10 mM inulin, pH 5.0) and incubated at 37 °C. Samples were taken immediately after mixing and after 3 h of incubation and stored at −20 °C. Thawed samples were centrifuged and the fructose content in the supernatant was determined by HPLC.

Results and Discussion

Construction and transfer of the mobilizable plasmid pKF4

Transformation, the common way to transfer new genes that are located on a plasmid, is not feasible with *A. eutrophus*, due to the lack of vectors with adequate transformation rates. Vectors such as pHS4 rarely have a transformation rate for *A. eutrophus* above the naturally-occurring mutation rate (Friehs, Unpubl.). Therefore

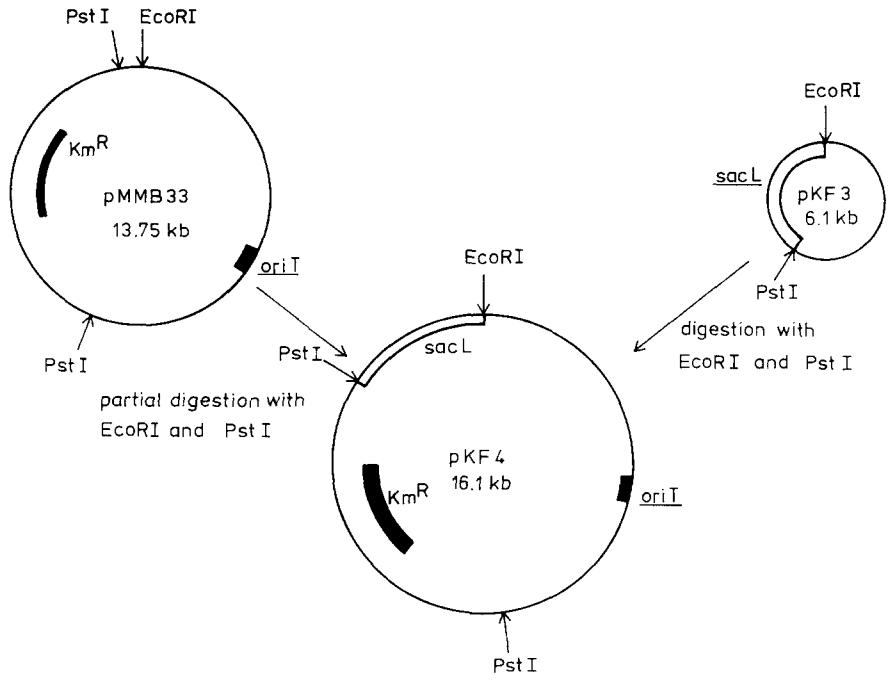

Fig. 1. Construction of plasmid pKF4. The plasmid pMMB33 was used to derive a DNA fragment with *Eco*RI/*Pst*I ends bearing the kanamycin resistance genes (Km) and the origin of conjugal transfer (oriT). This fragment was ligated with a 2.5 kb *Eco*RI/*Pst*I DNA fragment from plasmid pKF3 where the levanase gene was located (double lined segment). The resulting plasmid pKF4 was transferred into *Alcaligenes eutrophus* H16 using triparental conjugation technique with plasmid RP4-4 as helper plasmid.

it was necessary to use another transfer mechanism, namely a triparental conjugation system. The basic vector used was the mobilizable plasmid pMMB33, which requires a helper plasmid for safety reasons. In this case, we used RP4-4, a derivative of the plasmid RP4, as the helper plasmid (Hedges and Jacob, 1974). The plasmid pMMB33 possesses the *mob* region, which enables it to be mobilized, but not the transfer genes; they come from the helper plasmid. Therefore, pMMB33 cannot transfer itself to other organisms, an important safety consideration. RP4-4 can help to mobilize pMMB33, but cannot exist stably in the same host together with pMMB33.

The source for the levanase gene was the plasmid pKF3, described previously (Friehs et al., 1986). The plasmid pKF4 was constructed, as shown in Fig. 1, using common techniques and transformed into *E. coli* C600.

The triparental conjugation was done by mixing colonies of *A. eutrophus* H16, *E. coli* C600 pKF4, and *E. coli* J53 RP4-4 on a NB plate and incubating the mixture for 48 h at 30 °C. After that time, the mixture was spread out on H16 minimal medium plates containing sodium citrate as the carbon source and 150 mg l^{-1} kanamycin. Transconjugants were picked and screened for tetracyline sensitivity.

Tetracycline-sensitive colonies were plated on H16 minimal medium with sucrose as carbon source and 200 mg l^{-1} kanamycin. As a last screening test colonies were plated on special plates of Fluorocult ECD agar, (Merck, F.R.G.) to distinguish between *E. coli* and *A. eutrophus* clones. A second test was done with Kovacs indole reagent, according to the method of the supplier.

The difficulty of the screening was due to the fact that there was still little growth of *E. coli* on citrate minimal medium plates while the plasmid-free strain of *A. eutrophus* showed growth even at high kanamycin concentrations, due to its natural resistance. Therefore the kanamycin concentration was increased to 200 mg l^{-1}. Plating on ENDO C agar could not give clear screening results because of the *lacY* deletion in *E. coli* C600. The plating on Fluorocult medium provided clear evidence that the screened colonies were clones of *A. eutrophus*. The plasmid DNA from five clones was isolated and a restriction analysis was performed. The results showed that all five clones bore the plasmid pKF4.

Investigation of the expression of levanase in A. eutrophus H16 pKF4

The expression of the levanase genes was measured by the degradation of inulin

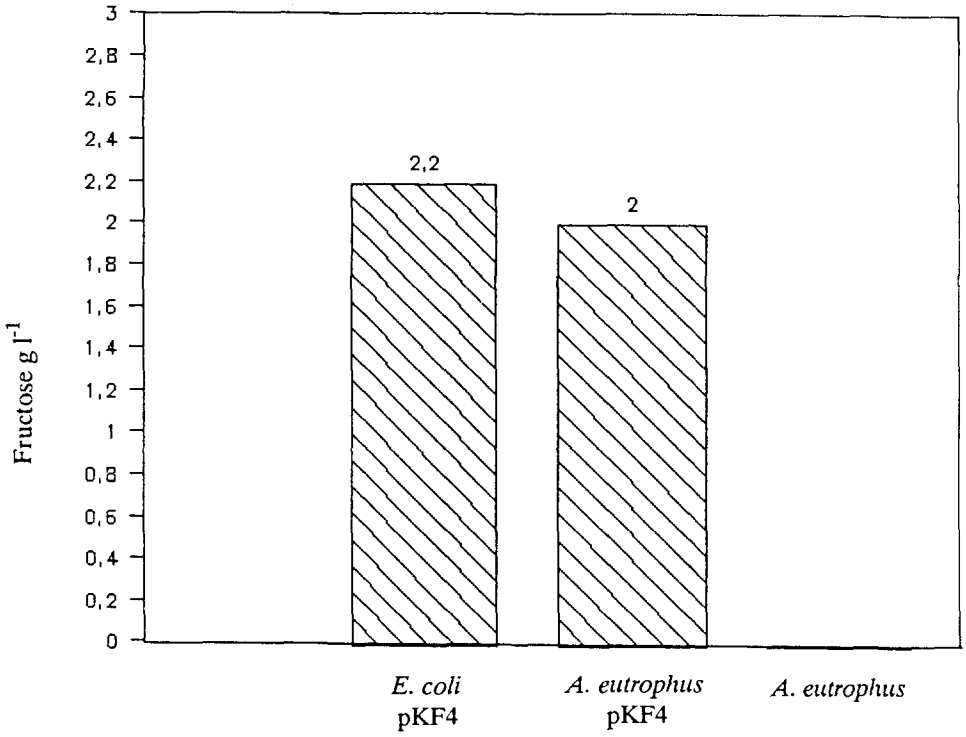

Fig. 2. Inulin degradation abilities of cell lysates of *E. coli* C600 containing the recombinant plasmid pKF4 and *Alcaligenes eutrophus* H16 with and without the plasmid pKF4 as measured by the formation of free fructose from inulin. Compared are lysates obtained from the same amount of cells referring to the optical density and after incubation with inulin assay buffer for 3 h.

by lysates of *A. eutrophus* H16 with and without the plasmid pKF4, as well as by those of *E. coli* C600 pKF4. Fig. 2 shows the comparison of the degradation of inulin by *A. eutrophus* H16 with and without the plasmid pKF4. With this assay, it could be shown that a *B. subtilis* gene under the control of a *B. subtilis* promoter was expressed in *A. eutrophus*. A comparison of the control sequence of the levanase with control sequences of *A. eutrophus* could show possible similarities. Obviously, the RNA polymerases of *A. eutrophus* are able to bind at *B. subtilis* DNA sequences.

Growth of A. eutrophus H16 pKF4 on sucrose-containing media

During the screening procedure, the clones were plated once on H16 minimal medium (200 mg l^{-1} kanamycin) with sucrose as the sole carbon source. The clones required several days to grow on these plates and the colonies never became large. It was assumed that this low growth was related with the kanamycin concentration. But growth was also low in submersed cultures using the same medium without kanamycin. Cells grew well in LB medium with sucrose, but the sucrose was used at a low rate. With this observation it became clear that there must be a major transport limitation; i.e. the sucrose is not transported into the system and/or the levanase is not secreted into the medium.

This work is a step to improve the substrate spectrum of *A. eutrophus* by using genetic engineering methods. Other genes for other enzymes could also be transferred, and it is conceivable to use strong, efficient, and perhaps controllable promoters from *A. eutrophus* itself. However, to improve the economics of PHB production the transport limitation problems have to be solved. Experiments to investigate these transport phenomenona and attempts to change the permeability of the membrane are in progress.

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