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CHARACTERIZATION OF THE PRECURSOR FORM OF THE EXOCELLULAR LEVANSUCRASE FROM BACILLUS SUBTILIS

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SUMMARY : Expression of the cloned levansucrase gene (sacB) was demonstrated in E. coli minicells by assay of the enzyme in crude extracts, SDS-polyacrylamide gel electrophoresis and immunoblotting. The existence of a precursor form of the enzyme of MW 53000 was also demonstrated and confirmed by the DNA sequence corresponding to the NH_2 terminal region of the protein.

The sucrose metabolic system is an interesting model for studying the regulation of gene expression and secretion in Bacillus subtilis. Biochemical and genetical studies have already been made on this system which comprises at least eight different loci (1). It includes three structural genes specifically induced by sucrose. One of them, sacB, codes for the exocellular levansucrase. Among the five known regulatory loci, four control the expression of sacB.

Levansucrase is a β-D-fructofuranosyl transferase (E. C. 2. 4. 1. 10) which synthesizes polymers of fructose called levans. It has been obtained in a pure state (2). It has a molecular weight of 50000 daltons ; its primary structure has been determined by protein sequencing (3) as well as its tertiary structure by X-ray diffraction analysis (4).

In this paper we describe the expression in an E. coli minicell producing strain of the protein corresponding to the structural gene sacB. The sequence of the signal peptide has been determined and the existence of a precursor of levansucrase has been demonstrated.

MATERIALS AND METHODS : E. coli SK1592 was used as a recipient strain when subcloning DNA fragments (5). The λ collection was constructed in E. coli KH802. E. coli was grown in L-medium. B. subtilis mutant QB2010 (sacA321 sacR37 sacU32 thr5) was constructed in this laboratory. Minicells of E. coli AR1062 were prepared as described by Rambach and Hogness (6). Incorporation of $|^{35}S|$ L-methionine was carried out according to the same authors. Transformation of E. coli and B. subtilis was performed as described before (7). Transformants were selected as previously reported (8, 9).

ABBREVIATIONS - Lvs : levansucrase, Lvs^h : hyperproduction of Lvs, Lvs^c : constitutive production of Lvs, PEA : phenylethyl alcohol

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E. coli recombinant plasmid DNA were purified according to the procedure of Birnboim and Doly (10) modified by Ish-Horowicz and Burke (11). DNA fractions were analyzed by electrophoresis on 0.5 % to 0.7 % agarose slab gels. Restriction fragments were recovered after electrophoresis by electro-elution in a dialysis bag.

Nucleotide sequences were determined by the method of Maxam and Gilbert (12). Labeling of the 5' ends of DNA fragments with $|\gamma^{32}P|$ ATP (3000 Cie/mmole, Amersham) and T4 polynucleotide kinase was performed by exchange reaction (12). Restriction fragments were labeled at their 3' ends with $|\alpha^{32}P|$ dNTP in the presence of DNA polymerase I according to Cossart and Gicquel-Sanzey (13).

Separation of proteins was performed on a 7.5 % to 15 % exponential gradient polyacrylamide gel according to O'Farrell (14). Immunoblotting was carried out as described by Bowen et al. (15). Rabbit anti-levansucrase antibodies were prepared as described by Kunst et al. (16). Levansucrase assay was performed according to Lepesant et al. (17).

RESULTS : Partial DNA nucleotide sequence of the sacB locus.

A library has been constructed in our laboratory by inserting sized partial Sau3A1 digest of DNA from strain QB2010 into the BamHI sites of the vector λ EMBL3 (18). One phage was obtained which contains the $sacR^c$ allele. Strains of B. subtilis carrying this mutation secrete Lvs constitutively. Comparing the different restriction sites obtained with those deduced from the known aminoacid sequence of the protein (the computer compilation was performed by G. Roizès), we concluded that this phage includes the entire sacB gene except for the last twenty six nucleotides.

An EcoRI-TaqI fragment of about 400 bp which overlaps the NH_2-terminal sequence of the protein and its putative signal sequence was subcloned into the large EcoRI-ClaI fragment of pBR322. The nucleotide sequence was determined starting from pBR322 EcoRI or HindIII sites by the method of Maxam and Gilbert (12). Results are reported on fig. 1. The deduced aminoacid sequence was compared to that obtained by protein sequencing by A. Delfour (3) and showed total identity downstream the Lys residue which is the mature protein N-terminal aminoacid.

Upstream this Lys residue there is an open reading frame of 29 aminoacids starting from an ATG codon. This sequence is characterized by the

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                                        -29
TAAAAAAGGA GACATGAACG ATG AAC ATC AAA AAG TTT GCA AAA CAA GCA ACG ATA
                      fMet Asn Ile Lys Lys Phe Ala Lys Gln Ala Thr Ile
                                                                  -1
 TTA ACC TTT ACT ACC GCA CTG CTG GCA GGA GGC GCA ACT CAA GCG TTT GCG |
 Leu Thr Phe Thr Thr Ala Leu Leu Ala Gly Gly Ala Thr Gln Ala Phe Ala |
+1
 AAA GAA ACG AAC CAA AAG CCA TAT AAG GAA ACA TAC GGC ATT TCC CAT ATT
 Lys Glu Thr Asn Gln Lys Pro Tyr Lys Glu Thr Tyr Gly Ile Ser His Ile

 ACA CGC CAT GAT ATG CTG CAA ATC CCC GAA CAG CAA AAA AAT GAA AAG
 Thr Arg His Asp Met Leu Gln Ile Pro Glu Gln Gln Lys Asn Glu Lys
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Fig. 1 : DNA and aminoacid sequences in the NH_2-terminal region of levansucrase.

The NH_2-terminal Lys of the mature levansucrase was taken as aminoacid + 1. The cleavage site between the signal sequence and the mature protein is indicated by a vertical bar.

presence of a hydrophilic region of 8 aminoacids with 3 Lys residues followed by a hydrophobic stretch of 21 residues with an Ala as last aminoacid. This kind of signal peptide seems to be classical, and similar ones have been already described for other exocellular enzymes in other Bacilli (19 - 22). It is interesting to note that 2 codons upstream from the ATG codon of the presumed precursor there is a TGA stop codon in the same reading frame. A good putative ribosome binding site is found 9 bp upstream the ATG with 8 bases out of 9 complementary to the 3' end of the 16S RNA of B. subtilis (23). We also sequenced, on one strand, about 185 nucleotides upstream the ATG initiation codon (data not shown). Analysis of this fragment showed no sequence homologous to those described for B. subtilis "- 35" and "- 10" vegetative promoter nucleotide sequences (23). However, we found an open reading frame which covers the entire sequence, in the same frame as that of levansucrase and its preceeding stop codon. The levansucrase gene could therefore be immediatly adjacent to another gene which has not been identified so far.

Expression of the sacB gene in E. coli minicells

To confirm the existence of the precursor and to determine its length, the sacB gene was expressed in an E. coli minicell producing strain. The plasmid pLS8, which contains the entire sacB gene, was obtained by D. Le Coq from a λ library (24). It was introduced by transformation into E. coli AR1062. Very few recombinants were obtained and the majority of them had lost the levansucrase activity. Two recombinant clones possessing the Lvs^+ phenotype detected directly on plate (8) were picked and further analyzed. The presence of an active levansucrase was confirmed by enzymatic assays on the sonicated extracts (the specific activity found was about 0.1 enzyme unit per mg bacterial protein). Furthermore, one of the clone recovered contained a plasmid with a DNA insertion located in the 0.95 kb HindIII fragment at the end of the sacB locus. This sequence is about 800 bp long and contains a PstI site ; it probably corresponds to IS1. The other clone harbored a plasmid identical to the original pLS8. The proteins of this latter clone were analyzed by SDS-PAGE after incorporation of $|^{35}S|$ L-methionine (Fig. 2). Several additional bands were detected compared to the pattern obtained for the extract of the cells containing the vector alone pBR325 (lane 1). One of them has a MW which corresponds to that of authentic levansucrase (50000), and another one has a MW of about 53000. Another gel was submitted to immunoblotting and it was observed that the same two bands reacted with anti-levanesucrase antibodies (Fig. 2B), suggesting that the lower polypeptide is the mature form and the larger one a precursor form of the enzyme. This was also demonstrated by adding PEA, a protein processing inhibitor (25) during the incorporation of methionine. The ratio of the 53000

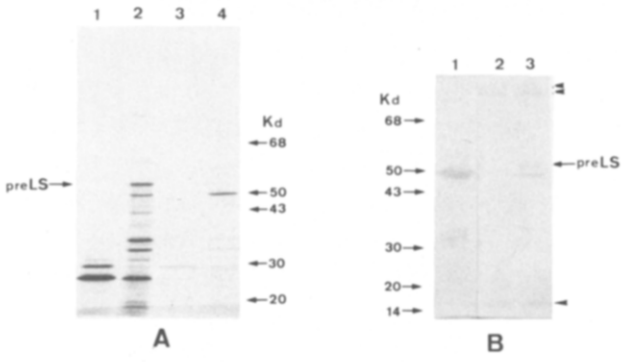

Fig. 2A : Analysis of the polypeptides coded by the plasmid pLS8 containing the sacB gene in E. coli AR1062.
Lane 1 : AR1062 (pBR325) ; lane 2 : AR1062 (pLS8) ; lanes 3 and 4 : supernatants of the minicells analyzed in lanes 1 and 2, respectively.
B : Immunodetection of the mature levansucrase and of the precursor of levansucrase (pre LS) after protein blotting.
Lane 1 : authentic levansucrase (2 µg) ; lane 2 : AR1062 (pBR325) ; lane 3 : AR1062 (pLS8).
Arrow-heads indicate the presence of non specific polypeptides.

to the 50000 dalton polypeptide was much higher in PEA treated cells than in the control (data not shown).

The supernatant of the non PEA treated labeled minicells was also subjected to electrophoresis (Fig. 2A lane 4). Only the mature form of levansucrase was detected, while no mature form of β-lactamase was observed in this case. This result may be considered as an argument for the secretion of the mature levansucrase in the E. coli minicell producing strain used. However this result seems to depend on the strain transformed by pLS8. Direct detection of the Lvs^+ phenotype on plates as reported before (8) could be carried out with AR1062 cells but a preliminary toluene treatment was necessary with SK1592 cells, indicating that the transformed minicell producing strain secreted more efficiently levansucrase than the transformed SK1592 cells. This suggests that the secretion of levansucrase observed in the minicells could be due to membrane defects of this division mutant strain.

DISCUSSION : Although heterospecific expression has been obtained for different genes from B. subtilis (20, 21, 26, 27) this has been difficult to achieve for the sacB gene. It seems that the presence of the cloned levansucrase gene has a deleterious effect in E. coli. This is examplified by the very low number of clones containing an active gene product in the minicell producing strain recovered after transformation with pLS8. It is also supported by the fact that one of these clones showing a Lvs^+ phenotype has a plasmid containing an insertion of 800 bp which probably corresponds to IS1. The few cells, which possess the intact recombinant plasmid, produced among

other polypeptides two components of 53000 and 50000 daltons. The larger one is probably the precursor of the mature levansucrase, which is normally secreted in B. subtilis. In this case the signal peptide must be about 30 aminoacid long. The supernatant of the minicells contained only the mature form of levansucrase, integrality of the cells being indicated by the absence of mature β-lactamase in these conditions. Preliminary results obtained with another E. coli strain using colorimetric test directly on plates showed that this phenomenon could be specific of the minicell producing strain. The secretion of levansucrase could therefore be ascribed to membrane defects of this strain although it is a specific secretion and not a leakage. The mature form of levansucrase has the same MW as that of the enzyme purified from the supernatant of the B. subtilis cultures, indicating that the recognition sites for processing of the precursor are similar in E. coli and in B. subtilis.

The sequence of the 5' end of the sacB gene has been determined and the aminoacid sequence of the signal peptide deduced. It contains 29 aminoacid residues and is characterized by the presence of a hydrophilic portion with 3 Lys residues followed by a hydrophobic sequence with an Ala residue in the - 1 position. This result is in agreement with the MW of the polypeptide expressed in the minicells. This signal sequence looks very much like others reported before in Bacilli (19 - 21). However the signal peptide of levansucrase appears smaller than that found for α-amylase, another secreted protein of B. subtilis (21, 22). The sequence of the NH_2-terminal portion of the mature levansucrase is in agreement with the results obtained by A. Delfour by aminoacid sequencing (3). The ATG codon corresponding to the Met residue located 29 aminoacids before the N-terminal Lys residue of the mature protein may be the start codon. This is supported by the fact that there is a TGA stop codon two codons upstream and in the same reading frame. A good putative ribosome binding site was also found 9 bp upstream from the ATG. Preliminary sequencing results indicate that the stop codon could correspond to the end of another gene since it is preceeded by an open reading frame of 185 bp. No promoter nucleotide consensus sequence was found in this sequence and it is therefore possible that the levansucrase is translated from a polycistronic mRNA. The levansucrase gene could therefore belong to an operon. This is being tested by determining the length of the corresponding RNA transcript and also by RNA polymerase binding experiments.

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REFERENCES

1. Lepesant, J-A., Kunst, F., Pascal, M., Kejzlarova-Lepesant, J., Steinmetz, M. and Dedonder R. (1976) In : Schlessinger D. (Ed.) Microbiology, Am. Soc. Microbiol. Washington DC, pp. 58-69.
2. Dedonder, R. (1966) Methods in Enzymol., 8, 500-505.
3. Delfour, A. (1981) Thèse de Doctorat d'Etat, Université Paris VII.
4. Lebrun, E. and Van Rapenbusch, R. (1980) J. Biol. Chem., 255, 12034-12036.
5. Bolivar, F. and Backman, K. (1979) Methods in Enzymol., 68, 245-267.
6. Rambach, A. and Hogness, D. (1977) Proc. Nat. Acad. Sci. U. S. A., 74, 5041-5045.
7. Rapoport, G., Klier, A., Billault, A., Fargette, F. and Dedonder, R. (1979) Molec. Gen. Genet., 176, 239-245.
8. Lepesant, J-A., Kunst, F., Lepesant-Kejzlarova, J. and Dedonder, R. (1972) Molec. Gen. Genet., 118, 135-160.
9. Dedonder, R. A., Lepesant, J-A., Lepesant-Kejzlarova, J., Billault, A., Steinmetz, M. and Kunst, F. (1977) Appl. Environ. Microbiol., 33, 989-993.
10. Birnboim, H. C. and Doly, J. (1979) Nucl. Ac. Res., 7, 1513-1523.
11. Ish-Horowicz, D. and Burke, J. F. (1981) Nucl. Ac. Res., 9, 2989-2998.
12. Maxam, A. M. and Gilbert, N. (1980) Methods in Enzymol., 65, 499-559.
13. Cossart, P. and Gicquel-Sanzey, B. (1982) Nucl. Ac. Res., 10, 1363-1378.
14. O'Farrell, P. H. (1975) J. Biol. Chem., 250, 4007-4021.
15. Bowen, B., Steinberg, J., Laemmli, U. K. and Weintraub, H. (1980) Nucl. Ac. Res., 8, 1-20.
16. Kunst, F., Pascal, M., Lepesant, J. A., Walle, J. and Dedonder, R. (1974) Eur. J. Biochem., 42, 611-620.
17. Lepesant, J-A., Billault, A., Kejzlarova, J., Pascal, M., Kunst, F. and Dedonder, R. (1974) Biochimie, 56, 1465-1470.
18. Murray, N. E., In : The bacteriophage λ. Vol. II, Cold Spring Harbor (in press).
19. Takkinen, K., Petterson, R. F., Kalkkinen, N., Palva, I., Söderlund, H. and Kääriäinen, L. (1983) J. Biol. Chem., 258, 1007-1013.
20. Kroyer, J. and Chang, S. (1981) Gene, 15, 343-347.
21. Yang, M., Galizzi, A. and Henner, D. (1983) Nucl. Ac. Res., 11, 237-249.
22. Yamazaki, H., Ohmura, K., Nakayama, A., Takeichi, Y., Otozai, K., Yamasaki, M., Tamura, G. and Yamane, K. (1983) J. Bacteriol., 156, 327-337.
23. Moran, C. P. Jr, Lang, N., Le Grice, S. F. J., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J. and Losick, R. (1982) Molec. Gen. Genet., 186, 339-346.
24. Gay, P., Le Coq, D., Steinmetz, M., Ferrari, E. and Hoch, J. A. (1983) J. Bacteriol., 153, 1424-1431.
25. Halegoua, S. and Inouye, M. (1979) J. Molec. Biol., 130, 39-61.
26. Fouet, A., Klier, A. and Rapoport, G. (1982) Molec. Gen. Genet., 186, 399-404.
27. Cantwell, B. A. and Mc Connell, D. J. (1983) Gene, 23, 211-219.