Journal of Industrial Microbiology, 4 (1989) 37–42
Elsevier

SIM 00156

Secretion of the sweet-tasting plant protein thaumatin by *Streptomyces lividans*

Charles Illingworth, Gregg Larson and Goran Hellekant

Department of Veterinary Science, University of Wisconsin, Madison, WI, U.S.A.

Received 30 March 1988
Revised 1 June 1988
Accepted 6 June 1988

Key words: Exported recombinant protein; Recombinant protein; β-Galactosidase; Leader peptide; Thaumatin

SUMMARY

To produce and direct the export in *Streptomyces lividans* of the sweet plant protein thaumatin, thaumatin II cDNA was fused in the correct reading frame to the β-galactosidase leader peptide, under the control of the β-galactosidase promoter and ribosome binding site. The export of the recombinant thaumatin may allow the correct formation of the thaumatin disulfide bonds. The recombinant thaumatin was purified from the medium on an S-Sepharose column and detected with western blots by sheep α-thaumatin antibodies. The recombinant thaumatin was the same size as authentic thaumatin and changed position on an acrylamide gel in response to reduction by 2-mercaptoethanol in the same manner.

INTRODUCTION

Thaumatin, a sweet protein from the arils of the fruits of the West African plant *Thaumatococcus danielli* Benth, is produced as a family of closely related sweet proteins (I, II, III [32], b and c [15]). Thaumatin is nearly 100 000 times sweeter than sucrose on a molar basis and is only detected by humans and old world monkeys, making thaumatin a useful tool in the study of taste in higher primates and man [12]. In *T. danielli* Benth, thaumatin is produced as a pre-pro-protein. The mature protein contains eight disulfide bonds, 207 amino acids, has an M_r value of 22 000 and an isoelectric point of pH 12 [20].

For the production of a cloned protein it is helpful to have the desired protein secreted into the medium. This allows a higher starting purity, avoids intracellular proteases, avoids interfering with intracellular metabolism and possibly favors the correct formation of disulfide bonds.

Streptomyces are soil bacteria which efficiently secrete a large number of proteins. *Streptomyces*

Correspondence: Goran Hellekant, Department of Veterinary Science, University of Wisconsin, Madison, WI 53703, U.S.A.

spp. remain viable, with active protein synthesis and production of secondary metabolites, well into stationary phase [4]. Over 40 years of industrial experience in the large-scale fermentative production of antibiotics by *Streptomyces* spp. and the progress in the molecular biology of the *Streptomyces* make them a useful potential host for the expression of heterologous proteins. *Streptomyces* spp. have been used as heterologous hosts for the expression of bovine growth hormone [13], human interleukin 2 [27] and *Escherichia coli* β-galactosidase [21].

Thaumatin has been expressed in *E. coli* [10], and in yeast [6,9,23]. We have attempted to produce thaumatin II in *S. lividans* which secretes β-galactosidase, an enzyme which has a 56 amino acid long leader peptide. To allow the sweet conformation of the recombinant thaumatin to form and to facilitate its recovery, the thaumatin II cDNA was placed in the correct reading frame ten amino acids after the *S. lividans* β-galactosidase leader peptide cleavage site, downstream of the β-galactosidase promoter and ribosome binding site. An extracellular thaumatin-like protein of the expected size was produced.

METHODS AND MATERIALS

Bacterial strains and plasmids. E. coli strain CAG1574 is a *recA*-derivative of MC1061 from Dr. Carol Gross, University of Wisconsin-Madison. *S. lividans* 66 [24], John Innes Institute stock No. 1326 was used.

pUR520 [33] contains the cDNA of mature thaumatin II in pBR322 and was provided by Dr. A.M. Ledeboer of Unilever Research Laboratories, The Netherlands. p3SSX-12 (Fig. 1) contains the *S. lividans* β-galactosidase promoter P3 and leader peptide [8] in a pBR322-pUC9 replicon and was provided by Dr. Dean Taylor of Smith Kline and French Laboratories, Philadelphia. p3SSX-12 has the sequence (in bold type) AAC **GCG CGG ATC**

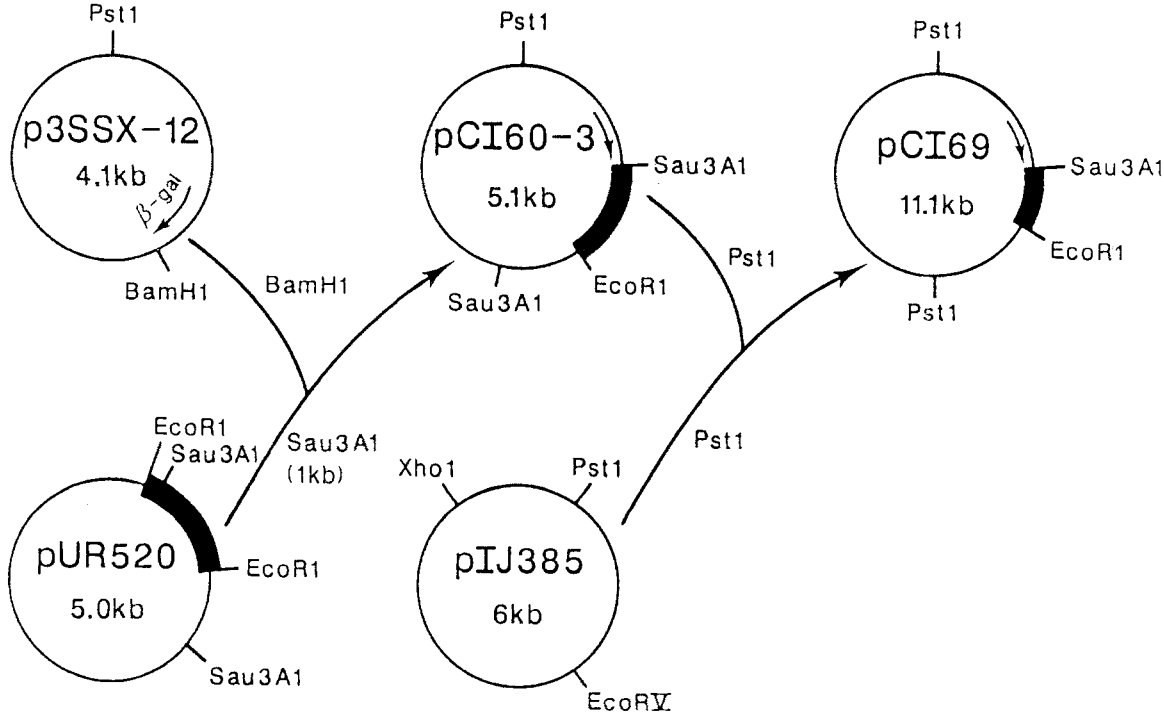

Fig. 1. The thaumatin II cDNA was fused to the β-galactosidase leader peptide and placed under the control of the β-galactosidase promoter and ribosome binding site, then placed in the *Streptomyces* high copy replicon pIJ385 as shown in the figure.

CGC G, providing a *Bam*HI site, situated eight amino acids past the normal leader peptide cleavage site [8]. pIJ385, a high copy number *Streptomyces* replicon derived from pIJ101 which confers thiostrepton resistance [17], was provided by Dr. H. Motamedi, University of Wisconsin-Madison.

Enzymes. Restriction enzymes, calf alkaline phosphatase and T4 DNA ligase were purchased from Bethesda Research Labs, New England Biolabs or Boehringer Mannheim Biochemicals. Each enzyme was used as specified by the manufacturer.

Isolation of plasmid DNAs. Plasmid DNAs were prepared by the rapid alkaline lysis method of Birnboim and Doly [3] and were purified by CsCl equilibrium centrifugation in the presence of ethidium bromide. Large DNA fragments and plasmids were separated by agarose gel electrophoresis and small DNA fragments were separated by polyacrylamide gel electrophoresis. For preparative purposes, DNA fragments in agarose gels were electroeluted onto NA-45 DEAE membrane (Schleicher and Schuell).

Transformation of E. coli. Competent *E. coli* was prepared and transformed by the method of Mandel and Higa [26].

Transformation of S. lividans. Protoplasts were produced and transformed by the method of Hopwood et al. [17].

Production and purification. S. lividans was grown in YEME [1] plus 34% sucrose, 1% galactose and 50 mM MOPS pH 7.0 containing thiostrepton (10 μg/ml) for 2–3 days at 28°C. The cells were discarded and the broth was treated with acetone (1:1.7) at 4°C. The precipitated proteins were washed on a 10 000-MW YM10 Amicon filter, run on an S-Sepharose fast flow column and eluted with a 0–0.25 M NaCl (0.02 M Tris-HCl pH 7.65) gradient [32].

Antiserum. Sheep antiserum raised against thaumatin was kindly provided by Dr. Henk Van der Wel, Unilever Research Labs, The Netherlands.

Western blot analysis. After electrophoresis in sodium dodecyl sulfate-12% polyacrylamide gels [22], thaumatin was visualized by the western blot method [30] with the use of sheep α-thaumatin serum, rabbit α-sheep serum and an ABC-AP kit (Vector Labs).

RESULTS

Construction of pCI69

The thaumatin II cDNA was placed in the *Streptomyces* expression vector p3SSX-12 as shown in Fig. 1. pUR520 was cleaved with *Sau*3A and the 1 kb fragment containing the mature thaumatin II cDNA [10,33] was gel-purified and ligated into *Bam*HI-cleaved p3SSX-12 to form pCI60-3 and transformed into *E. coli* CAG1574. pCI60-3 has the mature thaumatin II cDNA, minus its first four amino acids, fused in the correct reading frame to β-galactosidase downstream of the β-galactosidase ribosome binding site and promoter. The fusing to β-galactosidase occurs ten amino acids after the leader peptide cleavage site. pCI60-3 and the *Streptomyces* high copy number plasmid pIJ385 were both cleaved at their unique *Pst*I sites and ligated together to form pCI69 and transformed into *E. coli* CAG1574. *S. lividans* 1326 was then transformed with pCI69.

Production of thaumatin

S. lividans transformed with pCI69 and pIJ385 were grown in YEME plus sucrose and galactose

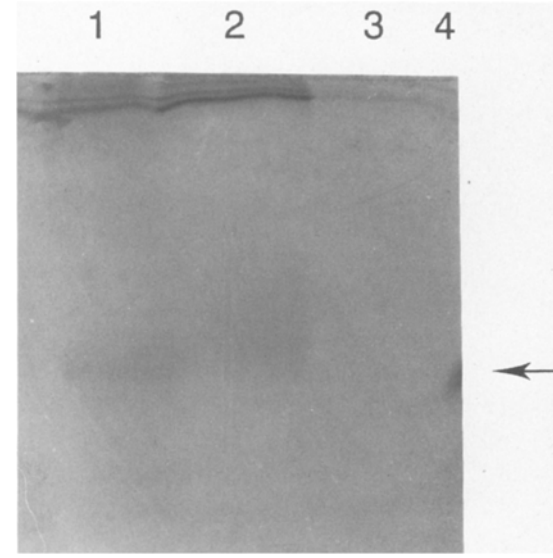

Fig. 2. A western blot using sheep α-thaumatin on a polyacrylamide gel of acetone-precipitated media (0.2 ml) of *S. lividans*–pCI69-2-2 (lane 1), *S. lividans*–pCI69-5-5 (lane 2), *S. lividans*–pIJ385 (lane 3) and thaumatin, 0.4 μg (lane 4). All samples were reduced with 2-mercaptoethanol.

and after 3 days the broths were analyzed on a polyacrylamide gel. The arrow in Fig. 2 indicates a band in the pCI69-2-2 and pCI69-5-5 lanes which is absent from the pIJ385 lane of a western blot probed with α-thaumatin sheep antibody. The yield of recombinant thaumatin is estimated to be 0.2 mg/l. A western blot probed with α-thaumatin sheep antibody showed cross-reactions (approximately ten bands) with the cell pellets from *S. lividans* containing pCI69-2-2, pCI69-5-5 and pIJ385, but no discernable difference was seen between pIJ385 and the pCI69 lanes (data not shown).

Purification of thaumatin

To further purify the recombinant thaumatin, 2 liters of *S. lividans* pCI69-2-2 were grown for 3 days. The cells were discarded, the media acetone-precipitated and the precipitate washed on a 10 000-MW cut-off Amicon filter and then loaded on an S-Sepharose column.

Following elution of the recombinant thaumatin from the S-Sepharose column at a position in the 0–0.25 M NaCl gradient expected for thaumatin ([32]; G. Larson, unpublished results), a western blot of a sample from the peak using the α-thaumatin sheep serum gave a positive reaction with a protein the same size as thaumatin (Fig. 3). When 2-mercaptoethanol was omitted from the loading buffer, the position, due to the eight cysteine disulfide cross-bridges in thaumatin, on the western blot of both thaumatin and the recombinant thaumatin shifted to an equivalent extent (data not shown). It is uncertain whether the recombinant thaumatin is in the correct conformation and sweet.

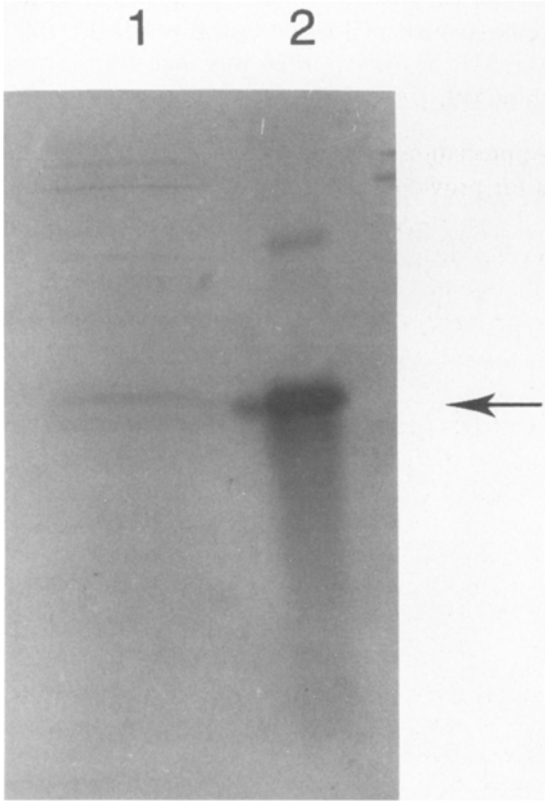

Fig. 3. A western blot using sheep α-thaumatin on a polyacrylamide gel of recombinant thaumatin purified from *S. lividans*–pCI69-2-2 media on an S-Sepharose column as described in the Methods section (lane 1) and thaumatin (lane 2). All samples were reduced with 2-mercaptoethanol.

DISCUSSION

Recombinant thaumatin was produced using the p3SSX-12 secretion vector system in *S. lividans* utilizing the promoter, ribosome binding site and leader peptide of the *S. lividans* β-galactosidase gene to express thaumatin II cDNA and to direct secretion of recombinant thaumatin. If the secondary structure of native thaumatin is disrupted or its eight cysteine disulfide bonds misform, the sweetness of thaumatin is lost [31]. The appropriate oxidation-reduction potential required for the correct pairing of cysteines is evidently not present in bacterial cytoplasm. In *T. danielli* Benth thaumatin is produced as a pre-pro-protein [10]. To mimic its natural production we have produced thaumatin as a pre-protein, using the *S. lividans* β-galactosidase leader peptide. The cDNA of thaumatin II in pUR520 lacks both the pre- and the pro- regions, containing only the mature thaumatin coding regions.

A number of exported *Streptomyces* proteins have been cloned and shown to contain leader peptides, including *S. lividans* β-galactosidase [4,8], *S. coelicolor* A3(2) agarase [2,5], *S. griseus* protease A and B [14], *S. hygroscopicus* α-amylase [18], *S. limosus* α-amylase [25], *S. plicatus* endoglycosidase H

[29] and *Streptomyces* R61 DD-peptidase [7]. To express thaumatin II in *S. lividans* the promoter and leader peptide of the *S. lividans* β-galactosidase was attached to the mature thaumatin II coding sequences lacking the first four amino acids in the correct reading frame ten amino acids after the leader peptide cleavage site. The four amino acids removed from the natural mature thaumatin II, Ala, Thr, Phe and Glu, are expected to be replaced by (1)Ala, (2)Asp, (3)Glu, (4)Pro, (5)Pro, (6)Glu, (7)Trp, (8)Asn, (9)Ala and (10)Arg. This replacement could affect the recombinant thaumatin's sweetness. The carboxy terminal pro- sequence of native thaumatin, removed during the processing of mature thaumatin, may be required for the formation of the correct sweet conformation of thaumatin. The lack of a sweet recombinant thaumatin may also be due to the low yields, with a small proportion of the molecules having the correct sweet conformation.

Western blots with α-thaumatin sheep antibody of the cell pellet of *S. lividans* containing pCI69 failed to detect the presence of recombinant thaumatin. This could be due to only low levels of unexported recombinant thaumatin being present or the dilution by cellular protein of the recombinant thaumatin. The export of the recombinant thaumatin provides a major enrichment for further purification and the high thaumatin isoelectric point (pH 12) allows a highly specific purification of the recombinant thaumatin via ion exchange chromatography.

The recombinant thaumatin was estimated to be produced at 0.2 mg/l. Other proteins expressed in *Streptomyces* using the β-galactosidase promoter and leader peptide of the p3SSX series of plasmids have produced protein at 2–10 mg/l (D. Taylor, personal communication). The low level of recombinant thaumatin production could be due to a number of causes, including lack of optimization of the fermentation conditions and media or proteolysis by the host strain *S. lividans* 1326. In Fig. 2 the recombinant thaumatin bands in lanes 1 and 2 look heterogeneous. The heterogeneous band could be caused by proteolysis, incorrect or noncleavage of the leader peptide, presence of a mixture of protein conformations with incomplete reduction by 2-mercaptoethanol or impurities in the broth sample (0.2 ml). Some mycelia started to lyse after 3 days of fermentation and may be a source of improperly processed recombinant thaumatin. The purification of the recombinant thaumatin using the S-Sepharose column gave a homogeneous band.

Streptomyces DNA has a 73% G + C base composition [11] and the codon usage in *Streptomyces* genes strongly favors codons with G or C in the third position. All 61 possible sense codons can be expressed by *Streptomyces* and it is not known whether the codon usage bias corresponds to the abundance of different tRNA species in *Streptomyces* [16]. The third position in the mature thaumatin II cDNA is 91.3% G + C and the thaumatin II cDNA shows a codon usage [9] more similar to *Streptomyces* [16] than, in decreasing order, *B. subtilis*, *E. coli* [28] or *Saccharomyces cerevisiae* [19].

ACKNOWLEDGEMENTS

Appreciation is extended to Unilever Laboratories for providing the thaumatin II cDNA and to Dr. Dean Taylor, Smith, Kline and French Laboratories, for providing p3SSX-12. We thank Dr. Henk Van der Wel and especially Dr. Jerry Ensign (Department of Bacteriology, University of Wisconsin-Madison) for invaluable assistance.

REFERENCES

1 Bibb, M.J., R.F. Freeman and D.A. Hopwood. 1977. Physical and genetical characterization of a second sex factor, SCP2, for *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. 154: 155–166.

2 Bibb, M.J., G.H. Jones, R. Joseph, M. Buttner and J.M. Ward. 1987. The agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2): affinity purification and characterization of the cloned gene product. J. Gen. Microbiol. 133: 2089–2096.

3 Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513–1523.

4 Burnett, W.V., M. Brawner, D.P. Taylor, L.R. Fare, J. Henner and T. Eckhard. 1985. Cloning and analysis of an exported β-galactosidase and other proteins from *Streptomyces lividans*, pp. 441–445. In: Microbiology-1985 (Leive, L., P. Bontventre, J.A. Morello, S. Schlesinger, S.D. Silver and H.C. Wu, eds.), American Society for Microbiology, Washington, DC.

5 Buttner, M., I.M. Fearnley and M.J. Bibb. 1987. The agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. Mol. Gen. Genet. 209: 101–109.

6 Date, T., M. Grunstein, S. Hollenberg, R. Koduri, J. Lai, J.-H. Lee and G. Wilcox. 1984. The manufacture and expression of genes for thaumatin. European Patent Application No. 0,139,501.

7 Duez, C., C. Piron-Fraipont, B. Joris, J. Dusart, M. Urdea, J. Martial, J.-M. Frère and J.-M. Ghuysen. 1987. Primary structure of the *Streptomyces* R61 extracellular DD-peptidase 1. Cloning into *Streptomyces lividans* and nucleotide sequence of the gene. Eur. J. Biochem. 162: 509–518.

8 Eckhardt, T., J. Strickler, L. Gorniak, W.V. Burnett and L.R. Fare. 1987. Characterization of the promoter, signal sequence, and amino terminus of a secreted β-galactosidase from *Streptomyces lividans*. J. Bacteriol. 169: 4249–4256.

9 Edens, L., I. Bom, A.M. Ledeboer, J. Maat, M.Y. Toonen, C. Visser and C.T. Verrips. 1984. Synthesis and processing of the plant protein thaumatin in yeast. Cell 37: 629–633.

10 Edens, L., L. Heslinga, R. Klok, A.M. Ledeboer, J. Maat, M.Y. Toonen, C. Visser and C.T. Verrips. 1982. Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*. Gene 18: 1–12.

11 Gladek, A. and J. Zakrzweska. 1984. Genome size of *Streptomyces*. FEMS Microbiol. Lett. 24: 73–76.

12 Glasser, D., G. Hellekant, J.N. Brouwer and H. Van der Wel. 1978. The taste responses in primates to the proteins thaumatin and monellin and their phylogenetic implications. Folia Primatol. 29: 56–63.

13 Gray, G., G. Selzer, G. Buell, P. Shaw, S. Escanez, S. Hofer, P. Voegeli and C. Thompson. 1984. Synthesis of bovine growth hormone by *Streptomyces lividans*. Gene 32: 21–30.

14 Henderson, G., P. Krygsman, C.J. Liu, C.C. Davey and L.T. Malek. 1987. Characterization and structure of genes for proteases A and B from *Streptomyces griseus*. J. Bacteriol. 169: 3778–3784.

15 Higginbotham, J.D. and C.A.M. Hough. 1977. Useful taste properties of amino acids and proteins. In: Sensory Properties of Foods (Birch, G.C., J.G. Brennan and K.J. Parker, eds.), pp. 129–149, Applied Sciences, London.

16 Hopwood, D.A., M.J. Bibb, K.F. Chater, G.R. Janssen, F. Malpartida and C.P. Smith. 1986. Regulation of gene expression in antibiotic-producing *Streptomyces*. In: Regulation of Gene Expression, 25 Years On – 39th Symposium of the Society for General Microbiology (Booth, I.R. and C.F. Higgins, eds.), Cambridge University Press.

17 Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward and H. Schrempf. 1985. Genetic Manipulation of *Streptomyces:* a Laboratory Manual. The John Innes Foundation, Norwich, U.K.

18 Hoshiko, S., O. Makabe, C. Nojiri, K. Katsumata, E. Satoh and K. Nagaoka. 1987. Molecular cloning and characterization of the *Streptomyces hygroscopicus* α-amylase. J. Bacteriol. 169: 1029–1036.

19 Ikemura, T. 1982. Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. J. Mol. Biol. 158: 573–597.

20 Iyengar, R.B., P. Smits, H. Van der Wel, F.J.G. Van der Ouderaa, J.H. Van Brouwershaven, P. Ravebstein, G. Richters and P.D. Van Wassenaar. 1979. The complete amino acid sequences of the sweet protein thaumatin I. Eur. J. Biochem. 96: 193–204.

21 King, A. and K. Chater. 1986. The expression of the *Escherichia coli lacZ* gene in *Streptomyces*. J. Gen. Microbiol. 132: 1739–1752.

22 Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227: 680–685.

23 Lee, J.-H., J. Lai and J. Weickmann. 1986. Production of food additives by genetically engineered microorganisms – cloning of thaumatin I and cecropin A genes in *Saccharomyces cerevisiae* and *Escherichia coli*. World Biotech. Rep. 2: 63–69.

24 Lomovskaya, N.D., N.M. Mkrtumian, N.L. Gostimskaya and V.N. Danilenko. 1972. Characterization of temperate actinophage φC31 isolated from *Streptomyces coelicolor* A3(2). J. Virol. 9: 258–262.

25 Long, C.M., M.-J. Virolle, S.-Y. Chang, S. Chang and M.J. Bibb. 1987. α-Amylase gene of *Streptomyces limosus:* nucleotide sequence, expression motifs, and amino acid sequence homology to mammalian and invertebrate α-amylases. J. Bacteriol. 169: 5745–5754.

26 Mandel, M. and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 154–162.

27 Muñoz, A., A. Perez-Aranda and J. Barbero. 1985. Cloning and expression of human interleukin 2 in *Streptomyces lividans* using the *Escherichia coli* consensus promoter. Biochem. Biophys. Res. Commun. 133: 511–519.

28 Ogasawara, N. 1985. Markedly unbiased codon usage in *Bacillus subtilis*. Gene 40: 145–150.

29 Robbins, P.W., R.B. Trimble, D.F. Wirth, C. Hering, F. Maley, G. Maley, R. Das, B. Gibson, N. Royal and K. Biemann. 1984. Primary structure of the *Streptomyces* enzyme endo-β-*N*-acetylglucosaminidase H. J. Biol. Chem. 259: 7577–7583.

30 Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and application. Proc. Natl. Acad. Sci. USA 76: 4350–4354.

31 Van der Wel, H. 1974. In: Symposium Sweeteners (Inglett, G., ed.), pp. 194–203, AVI Publishing Co., Westport, CT.

32 Van der Wel, H. and K. Loeve. 1972. Isolation and characterization of thaumatin I and II, the sweet tasting proteins from *Thaumatococcus danielli* Benth. Eur. J. Biochem. 31: 221–225.

33 Verrips, C.T., A.M. Ledeboer, L. Edens, R. Klok and J. Maat. 1981. DNA sequences encoding the various allelic forms of mature thaumatin, and cloning vehicles comprising said DNA's and their use in transforming microorganisms. European Patent Application No. 54,330.