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HYBRID BACILLUS ENDO-(1-3,1-4)-β-GLUCANASES: CONSTRUCTION OF RECOMBINANT GENES AND MOLECULAR PROPERTIES OF THE GENE PRODUCTS

by

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Hybrid β-glucanase genes were constructed by the reciprocal exchange of the two halves of the isolated β-glucanase genes from Bacillus amyloliquefaciens and B. macerans. The β-glucanase hybrid enzyme 1 (H1) contains the 107 amino-terminal residues of mature B. amyloliquefaciens β-glucanase and the 107 carboxyl-terminal amino acid residues of B. macerans β-glucanase. The reciprocal β-glucanase hybrid enzyme 2 (H2) consists of the 105 amino-terminal residues from the B. macerans enzyme and the carboxyl-terminal 107 amino acids from B. amyloliquefaciens. The biochemical properties of the two hybrid enzymes differ significantly from each other as well as from both parental β-glucanases.

Hybrid β-glucanase H1 exhibits increased thermostability in comparison to other β-glucanases, especially in an acidic environment. This hybrid enzyme has maximum activity between pH 5.6 and 6.6, whereas the pH-optimum for enzymatic activity of B. amyloliquefaciens β-glucanase was found to be at pH 6 to 7 and for B. macerans at pH 6.0 to 7.5. Hybrid enzyme 1 being more heat stable than both parental enzymes represents a case of intragenic heterosis.

Hybrid β-glucanase 2 (H2) was found to be more thermolabile than the naturally occurring β-glucanases it was derived from and the pH-optimum for enzymatic activity was determined to be between pH 7 and pH 8.

1. INTRODUCTION

The mixed linked (1-3,1-4)-β-glucans constitute the major part of the endosperm cell walls of cereals like oat and barley. They can cause severe problems in the brewing industry such as reduced yield of extract and lowered rates of wort separation or beer filtration. Remaining β-glucans in the finished beer may lead to formation of hazes and gelatinous precipitates (11). Barley (1-3,1-4)-β-glucanases (EC 3.2.1.73) are synthesized in the scutellum and the aleurone layer during the early stages of germi-

Abbreviations: aa = amino acids; amp = ampicillin; bgl = β-glucanase gene; β-glucan = (1-3,1-4)-β-D-glucan; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; tet = tetracycline; SP = signal peptide.

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nation (21). However, a large proportion of the malt β-glucanase is irreversibly heat inactivated during kilning and the remaining activity is rapidly destroyed during mashing (18). Therefore, thermostable (1-3,1-4)-β-glucanases of fungal or bacterial origin are often added during mashing.

The best characterized bacterial (1-3,1-4)-β-glucanases are those from Bacillus subtilis and B. amyloliquefaciens and the genes encoding these enzymes have been cloned and sequenced (6, 9, 14, 23). It has recently been shown that the β-glucanase from B. macerans is more thermostable than the B. subtilis and B. amyloliquefaciens enzymes (4, 5). The B. macerans β-glucanase gene has been cloned (7) and its nucleotide sequence determined (BORRISS et al., in prep.). Comparison of the derived amino acid sequence with the derived sequences of B. subtilis and B. amyloliquefaciens discloses an overall homology of 70%.

In the present report we describe the construction of hybrid bacterial β-glucanase genes encoding active enzymes that degrade barley β-glucan and lichenan. Analyses of the biochemical properties of the hybrid enzymes in comparison to the parental β-glucanases are presented.

2. MATERIALS AND METHODS

2.1. Strains, plasmids and growth media

E. coli DH5α cells: F⁻, *endA1, hsd R17* (r_k^-, m_k^+), *supE44, thi1,* λ⁻, *recA1, gyrA96, relA1, ø80dlacZ,* Δ*M15* (12) were used for propagation of plasmids and for expression of β-glucanase genes. The vectors comprised pBR322 (2) and pUC19 (30). The recombinant plasmid pEG1 (6) carries an insert with the B. amyloliquefaciens β-glucanase gene and pUC13-Mac carries a DNA insert with the β-glucanase gene from B. macerans which is identical to the insert of

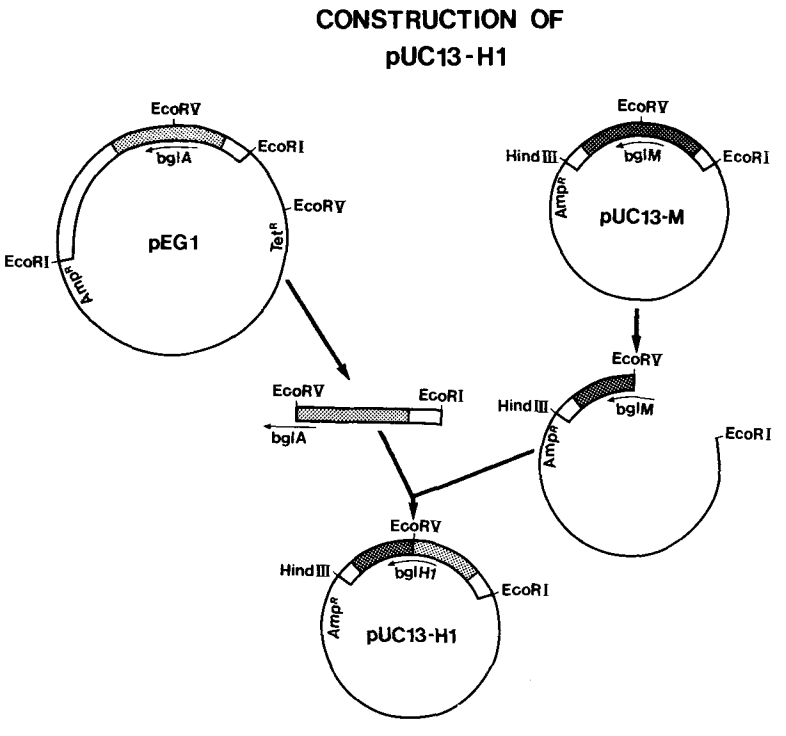

Figure 1. Construction of an E. coli expression and secretion vector containing the hybrid gene bgl-H1. bgl-A: (1-3,1-4)-β-glucanase gene from B. amyloliquefaciens. bgl-M: (1-3,1-4)-β-glucanase gene from B. macerans. For details, see section 2.5.

plasmid pUC19/34 (7). Media and growth conditions were as described previously (7).

2.2. Enzymes and chemicals

Radioactive nucleotides were from New England Nuclear, Boston, Massachusetts, USA. Restriction endonucleases, calf intestinal phosphatase and T4-DNA ligase were from Boehringer Mannheim, Mannheim, W. Germany. Modified T7-DNA polymerase (Sequenase™) was from United States Biochemical Corporation, Cleveland, Ohio, USA. A Geneclean™ kit was from BIO 101 Inc., La Jolla, California, USA. Barley β-glucan as well as a β-glucanase assay kit was purchased from Biocon, Boronia, Victoria, Australia. Lichenan was prepared from Cetraria islandica as described previously (3).

2.3. Transformation

E. coli cells were grown and prepared for transformation as described by LEDERBERG and COHEN (17) and the competent cells were stored frozen as described by THOMSEN (29).

2.4. DNA purification

Plasmid DNA was prepared from E. coli by the method of HATTORI and SAKAKI (13). Specific DNA fragments generated by restriction endonuclease digestion were separated by agarose gel electrophoresis and purified from the gel matrix using a Geneclean™ kit according to the manufacturer's recommendations.

2.5. Construction of hybrid β-glucanase genes

The B. amyloliquefaciens and B. macerans β-glucanase genes, and proteins, are highly homologous. In the center of the genes is a unique EcoRV restriction site which was used as fusion point in the construction of hybrid β-glucanase genes.

Construction of pUC13-H1 (Fig. 1): An EcoRV fragment which contains the 5'-flanking region and the amino-terminal half coding region of the B. amyloliquefaciens β-glucanase gene was isolated from plasmid pEG1 (6) and ligated with the large EcoRV-EcoRI fragment from pUC13-M encoding the carboxyl-terminal half of the B. macerans enzyme thus generating plasmid pUC13-H1 carrying the hybrid gene bgl-H1. E. coli DH5α cells transformed with pUC13-H1 are resistant to ampicillin.

Construction of pUC19-H2 (Fig. 2): For construction of the reciprocal recombinant gene, the B. macerans β-glucanase gene was excised as an EcoRI-PstI fragment from plasmid pUC13-M and recloned in pBR322 giving rise to plasmid pBR-MAC1 from which the small EcoRV fragment was purified and fused to the large EcoRV fragment from plasmid pEG1. With the insert in the correct orientation the plasmid is designated pEG-H2 and E. coli cells transformed with the plasmid were selected on medium containing tetracycline. The hybrid gene was excised from pEG-H2 as an EcoRI-BglII fragment and recloned in EcoRI-BamHI digested pUC19 to give plasmid pUC19-H2.

2.6. DNA sequence determination

Modified T7-DNA polymerase (Sequenase™) was used for nucleotide sequence determination around splice junctions of hybrid β-glucanase genes. The reactions were performed as described by ZHANG et al. (32).

2.7. Enzyme purification and analysis

For determination of thermostability of the hybrid enzyme H1 and parental enzymes E. coli cells harbouring the plasmid pUC13-H1, the plasmids pEG1 and pUC13-M were grown in tryptone-yeast medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per l) at 37 °C for 16 to 20 hours. The cells were lysed by sonication (Branson Sonifier) and after clearing of the lysate by centrifugation β-glucanase stability was analysed by incubation of the reaction mixture containing an aliquot of clarified lysate for various length of time at 65 °C or 70 °C followed by determination of residual β-glucanase activity.

Purification of β-glucanase from cell extracts as described in (7) has been used for the parental enzymes and hybrid enzyme H1. Due to low

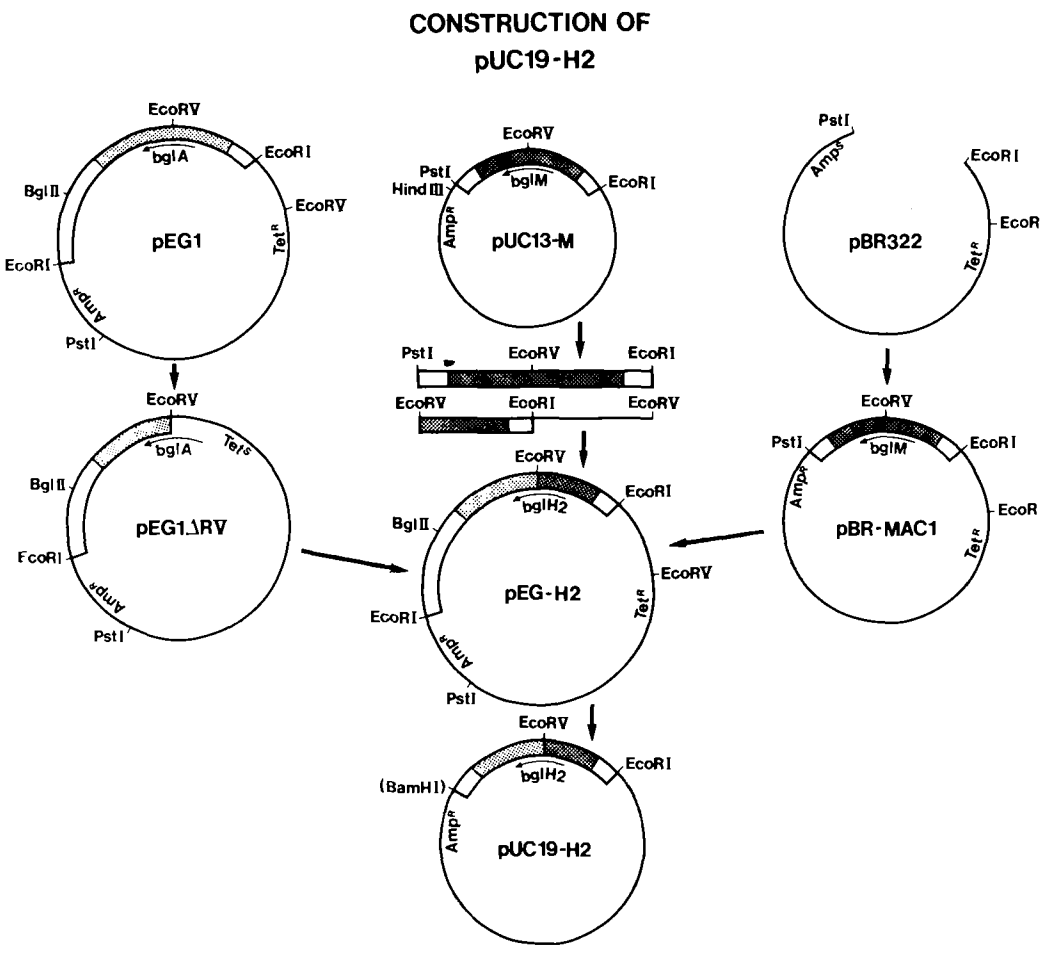

Figure 2. Construction of an E. coli expression and secretion vector containing the hybrid gene bgl-H2. bgl-A: (1-3,1-4)-β-glucanase gene from B. amyloliquefaciens. bgl-M: (1-3,1-4)-β-glucanase gene from B. macerans. For details, see section 2.5.

yield of H2 β-glucanase this enzyme was not purified to homogeneity. Ammonium sulphate precipitation of crude cell extracts enriched this β-glucanase to a specific activity of 10.4 U/mg (10.4 μmole glucose $mg^{-1} \cdot min^{-1}$). Protein concentration was determinated according to BRADFORD (8) using bovine serum albumin as standard. Enzyme preparations were analysed by SDS-PAGE (16).

β-glucanase assays:

Method A: The reaction mixture consisted of 1 ml 0.5% (w/v) lichenan or barley β-glucan in 40 mM Na-acetate buffer, pH 6.0, with or without

Figure 3. Structure of the bgl-H1 gene. **a**, Nucleotide sequence of the bgl-H1 gene and derived amino acid sequence of the hybrid pre-protein consisting of the signal peptide and the amino-terminal of the B. amyloliquefaciens protein and the carboxyl-terminal half of the B. macerans β-glucanase. The EcoRV site used for splicing is indicated. An arrow indicates the signal peptidase cleavage site. **b**, Diagram of the bgl-H1 gene and details of the fusion region. SP: signal peptide.

a

```
EcoRI
                    30                  60                  90
GAATTCAACGAAGAATCGCTGCACTATTATCGATTCGTCACCCACTTAAAGTTTTTCGACCAGCGTCTTTTTAACGGCACACACATGGAA

                   120                 150                 180
AGCCAGGACGATTTTTTACTGGAGACAGTGAAAGAAAAGTATCATCAGGCGTATAAATGCACGAAGAATATCCATACCTACATTGAGAAA

                   210                 240                 270
GAGTATGGGCATAAGCTCACCAGTGACGAGCTGCTGTATTTAACGATTCACATAGAAAGGGTAGTCAAACAAGTATAATGAAAGCGCTTT

                   300                 330                 360
CCTCGTATTAATTGTTTCTTCCATTCATATATAGGATTGTTACGGATAAAGCAGGCAAAACCTATCTGTCTGTGCTGATGGTAGTTTAGG

                   390                 420                 450
TTTGTATTTTTAACAGAAGGATTATCATTATTTCGACCGATGTTCCCTTTGAAAAGGATCATGTATGATCAATAAAGAAAGCGTGTTCAA

                   480                 510                 540
AAAAGGGGGAATGCTAACATGAAACGAGTGTTGCTAATTCTTGTCACCGGATTGTTTATGAGTTTGTGTGGGATCACTTCTAGTGTTTCG
                MetLysArgValLeuLeuIleLeuValThrGlyLeuPheMetSerLeuCysGlyIleThrSerSerValSer

                   570                 600                 630
GCTCAAACAGGCGGATCGTTTTTTGAACCTTTTAACAGCTATAACTCCGGGTTATGGCAAAAAGCTGATGGTTACTCAAATGGAGATATG
AlaGlnThrGlyGlySerPhePheGluProPheAsnSerTyrAsnSerGlyLeuTrpGlnLysAlaAspGlyTyrSerAsnGlyAspMet
 ↑
                   660                 690                 720
TTTAACTGCACTTGGCGTGCTAATAACGTCTCTATGACGTCATTAGGTGAAATGCGTTTGGCGCTGACAAGTCCGTCTTATAACAAGTTT
PheAsnCysThrTrpArgAlaAsnAsnValSerMetThrSerLeuGlyGluMetArgLeuAlaLeuThrSerProSerTyrAsnLysPhe

                   750                 780                 810
GACTGCGGGGAAAACCGCTCGGTTCAAACATATGGCTATGGACTTTATGAAGTCAGAATGAAACCGGCTAAAAACACAGGGATTGTTTCA
AspCysGlyGluAsnArgSerValGlnThrTyrGlyTyrGlyLeuTyrGluValArgMetLysProAlaLysAsnThrGlyIleValSer

                   840          EcoRV 870                 900
TCGTTCTTCACTTATACAGGTCCAACGGAGGGGACTCCTTGGGATGAGATTGATATCGAATTTCTAGGAAAAGACACGACAAAAGTCCAG
SerPhePheThrTyrThrGlyProThrGluGlyThrProTrpAspGluIleAspIleGluPheLeuGlyLysAspThrThrLysValGln

                   930                 960                 990
TTTAACTATTATACCAATGGGGTTGGCGGTCATGAAAAGGTTATCTCTCTTGGCTTTGATGCATCAAAGGGCTTCCATACCTATGCTTTC
PheAsnTyrTyrThrAsnGlyValGlyGlyHisGluLysValIleSerLeuGlyPheAspAlaSerLysGlyPheHisThrTyrAlaPhe

                  1020                1050                1080
GATTGGCAGCCAGGGTATATTAAATGGTATGTAGACGGTGTTTTGAAACATACCGCCACCGCGAATATTCCGAGTACGCCAGGCAAAATT
AspTrpGlnProGlyTyrIleLysTrpTyrValAspGlyValLeuLysHisThrAlaThrAlaAsnIleProSerThrProGlyLysIle

                  1110                1140                1170
ATGATGAATCTATGGAACGGAACCGGAGTGGATGACTGGTTAGGTTCTTATAATGGAGCGAATCCGTTGTACGCTGAATATGACTGGGTA
MetMetAsnLeuTrpAsnGlyThrGlyValAspAspTrpLeuGlySerTyrAsnGlyAlaAsnProLeuTyrAlaGluTyrAspTrpVal

                  1200                1230  HindIII
AAATATACGAGCAATTAATATGATTGCAGCTGGGCATGAGCTTTTTAGTCCACTCCAGGCATGCAAGCTT
LysTyrThrSerAsn
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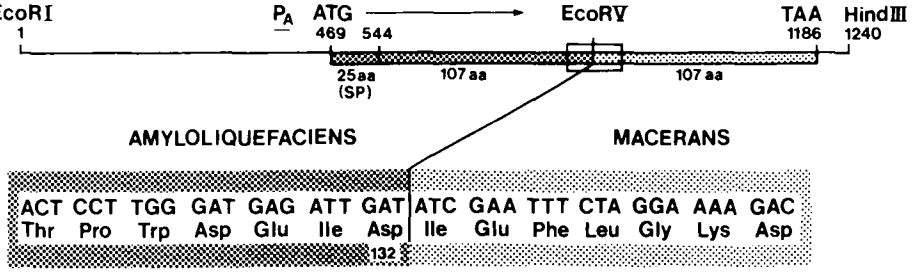
b HYBRID GENE ENCODING(1-3,1-4)-β-GLUCANASE H1



a

```
EcoRI                30                  60                  90
GAATTCCAGCTCGGATATACTATAATTACCCAGGTAAAATATTCCAACACCGTGGCTCCATAACTTCGTTCATATTTAAAATCATTTTGG

                120                 150                 180
AGGTGTATTATGAAAAAGAAGTCCTGTTTTACACTGGTGACCACATTTGCGTTTTCTTTGATTTTTTCTGTAAGCGCTTTAGCGGGGAGT
        MetLysLysLysSerCysPheThrLeuValThrThrPheAlaPheSerLeuIlePheSerValSerAlaLeuAlaGlySer
                                                                    ↑
                210                 240                 270
GTGTTCTGGGAACCATTAAGTTATTTTAATCCGAGTACATGGGAAAAGGCAGATGGGTATTCCAATGGGGGGGTGTTCAATTGCACATGG
ValPheTrpGluProLeuSerTyrPheAsnProSerThrTrpGluLysAlaAspGlyTyrSerAsnGlyGlyValPheAsnCysThrTrp

                300                 330                 360
CGTGCCAACAATGTTAATTTTACGAATGATGGAAAGCTCAAGCTGGGCTTAACGAGTTCTGCGTACAACAAATTTGACTGCGCGGAGTAC
ArgAlaAsnAsnValAsnPheThrAsnAspGlyLysLeuLysLeuGlyLeuThrSerSerAlaTyrAsnLysPheAspCysAlaGluTyr

                390                 420                 450
CGATCAACGAACATTTACGGATACGGCCTGTACGAGGTCAGTATGAAGCCAGCCAAAAATACAGGAATTGTCTCATCCTTTTTCACGTAT
ArgSerThrAsnIleTyrGlyTyrGlyLeuTyrGluValSerMetLysProAlaLysAsnThrGlyIleValSerSerPhePheThrTyr

                480  EcoRV          510                 540
ACAGGACCTGCTCATGGCACACAATGGGATGAAATAGATATCGAATTTTTGGGAAAAGACACAACGAAGGTTCAATTTAACTATTATACA
ThrGlyProAlaHisGlyThrGlnTrpAspGluIleAspIleGluPheLeuGlyLysAspThrThrLysValGlnPheAsnTyrTyrThr

                570                 600                 630
AATGGCGCAGGAAACCATGAGAAGTTCGCGGATCTCGGATTTGATGCAGCCAATGCCTATCATACGTATGCGTTCGATTGGCAGCCAAAC
AsnGlyAlaGlyAsnHisGluLysPheAlaAspLeuGlyPheAspAlaAlaAsnAlaTyrHisThrTyrAlaPheAspTrpGlnProAsn

                660                 690                 720
TCTATTAAATGGTATGTCGATGGGCAATTAAAACATACTGCAACAACCCAAATACCGGCAGCGCCGGGGAAAATCATGATGAATTTGTGG
SerIleLysTrpTyrValAspGlyGlnLeuLysHisThrAlaThrThrGlnIleProAlaAlaProGlyLysIleMetMetAsnLeuTrp

                750                 780                 810
AATGGTACGGGTGTTGATGATTGGCTCGGTTCCTACAATGGCGTAAATCCGATATACGCTCATTACGACTGGATGCGCTATAGAAAAAAA
AsnGlyThrGlyValAspAspTrpLeuGlySerTyrAsnGlyValAsnProIleTyrAlaHisTyrAspTrpMetArgTyrArgLysLys

                840                 870                2300
TAATGTACAGAAAAGGATTTCGCTGGCGGAATCCTTTTTTGATTAAAACGAAATAATCCC.....................AGATCT
                                                                                    BglII
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b HYBRID GENE ENCODING(1-3,1-4)-β-GLUCANASE H2

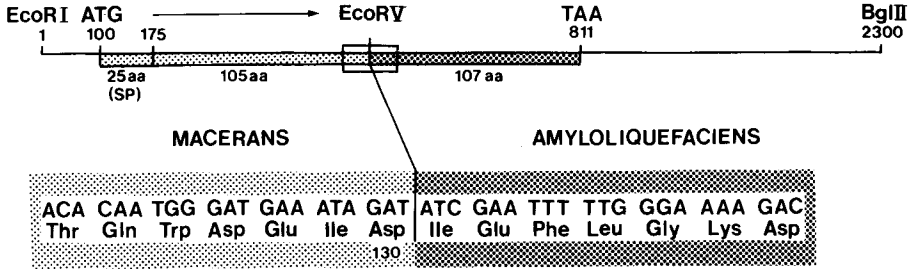

Figure 4. Structure of the bgl-H2 gene. **a**, Nucleotide of the bgl-H2 gene and derived amino acid sequence of the hybrid pre-protein consisting of the signal peptide and the amino-terminal half of the B. macerans protein and the carboxyl-terminal half of the B. amyloliquefaciens protein. The EcoRV site used for splicing is indicated. An arrow indicates the signal peptidase cleavage site. The sequence of the 3' non-coding region is not shown. **b**, Diagram of the bgl-H2 gene and details of the fusion region. SP: signal peptide.

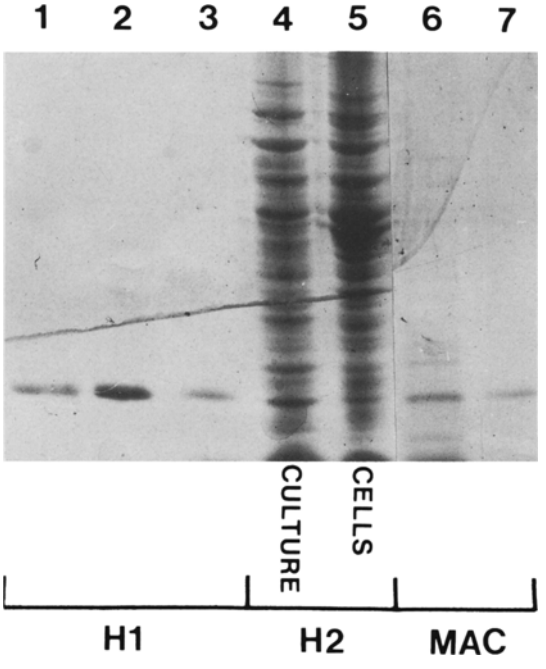

Figure 5. SDS-PAGE of samples containing hybrid β-glucanases and B. macerans β-glucanase. Lanes 1-3: 2 μg, 5 μg, and 1 μg purified β-glucanase H1. Lane 4: sample containing 50μg supernatant protein and lane 5: 100 μg cell extract of E. coli cells transformed by pUC-H2. Lane 6: 2 μg of partially purified B. macerans β-glucanase. Lane 7: 1 μg of purified B. macerans β-glucanase.

10 mM $CaCl_2$. The reaction was initiated by addition of 0.1 ml enzyme solution and incubation was at 37 °C for 20 min. The reaction was stopped by addition of 0.5 ml 3,5-dinitrosalicylic acid and the amount of reducing sugars were measured using the reagent formulation outlined by MILLER (22). Specific activity is expressed as μmole glucose released per min and mg of protein. Method B: Alternatively, azo-barley β-glucan was used as substrate for analysis of β-glucanase activity (20). The buffers employed were: 40 mM sodium acetate, pH 3.6-5.6; 40 mM potassium-sodium phosphate, pH 6-8; 40 mM Tris-HCl, pH 8.4-8.8.

Plate assay: E. coli cells were incubated on solid medium containing 0.2% (w/v) lichenan. Staining with 0.2% (w/v) Congo red reveals a clearing zone around colonies expressing β-glucanase.

2.8. Containment

All experiments involving recombinant DNA were carried out under BL1 laboratory conditions and waste containing biological material was autoclaved.

3. RESULTS

3.1. Hybrid β-glucanase genes

The fragment for the expression of the bgl-H1 recombinant gene is shown in Figure 3. The construct contains 469 bp of the flanking region, 75 bp encoding the signal peptide, and 321 bp encoding the amino-terminal half of the B. amyloliquefaciens β-glucanase. This 865 bp DNA stretch is fused in frame to the carboxyl-terminal half coding region as well as 51 bp of the 3'-flanking region of the β-glucanase gene from B. macerans.

The other recombinant gene, bgl-H2, (Fig. 4) consists of 99 bp of the 5'-flanking region, 75 bp encoding the signal peptide and 315 bp encoding the amino-terminal half of the B. macerans β-glucanase. This 489 bp fragment is fused in frame to a 321 bp DNA segment encoding the

Table I. Synthesis of β-glucanase in E. coli cells transformed with pUC13-H1 and pUC19-H2, respectively

plasmid	β-glucanase activity (μmole glucose ml culture^{-1} · min^{-1}) cells	supernatant
pUC13-H1	67.5	7.0
pUC19-H2	0.06	n.d.

n.d. = not detectable

Cells were grown in tryptone-yeast medium with intensive shaking for 20 h at 37 °C. After centrifugation (5000×g, 10 min), the supernatant was used directly for assay of enzyme activity. The pellet was washed, resuspended in 40 mM acetate, pH 6 and sonicated on ice 4×20 sec with a Branson Sonifier and clarified by centrifugation.

Table II. Kinetic parameters of hybrid and parental β-glucanases

β-glucanase	hybrid 1	hybrid 2	macerans	amyloliquefaciens
Relative V_{max}				
Glucan	1	1	1	1
Lichenan	0.77	0.88	0.73	1.1
K_m (mg/ml)				
Glucan	1.25	1.67	0.83	1.25
Lichenan	1.05	1.54	0.67	1.67
Specific activity (μmole glucose $mg^{-1} \cdot min^{-1}$)	3722	10.4*	5030	1330 (5)

* enriched cell extract

carboxyl-terminal half of B. amyloliquefaciens β-glucanase and approximately 1.5 Kb 3'-flanking region.

Plasmid constructions were analysed by restriction enzyme digests, DNA sequence determination around splice junctions, or both.

3.2. Analysis of hybrid gene products

Hybrid β-glucanase genes were expressed in E coli cells and the hybrid β-glucanase H1 wa purified according to the procedure used for B macerans β-glucanase (7). By SDS-PAGE it wa confirmed that the β-glucanase migrated as on

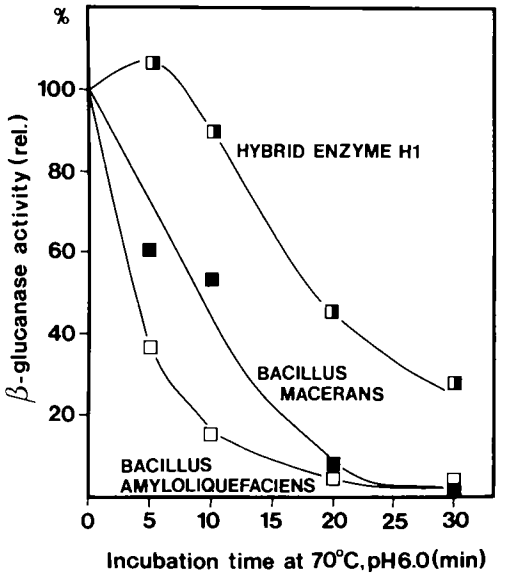

Figure 6. Activity of Bacillus hybrid β-glucanase H1 and parental enzymes in crude extracts (2.7) from transgenic E. coli cells after incubation for various lengths of time at 70 °C, pH 6.0. Activity is expressed as per cent of activity at time 0.

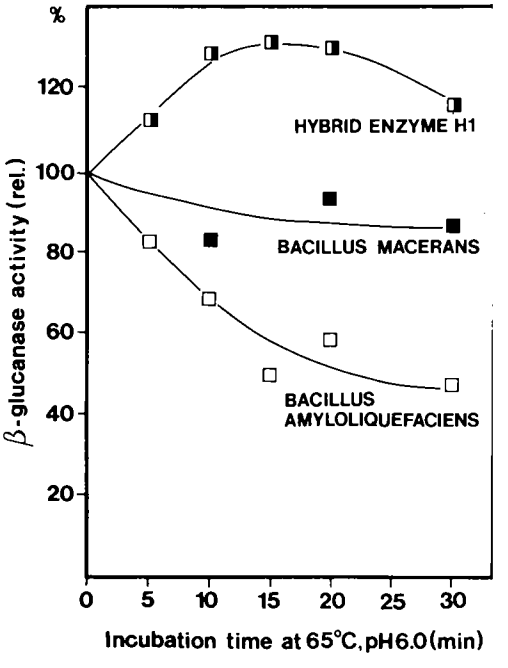

Figure 7. Activity of hybrid Bacillus β-glucanase H1 and parental enzymes in crude extracts (2.7) from transgenic E. coli cells after incubation for various lengths of time at 65 °C, pH 6.0. Activity is expressed as per cent of activity at time 0.

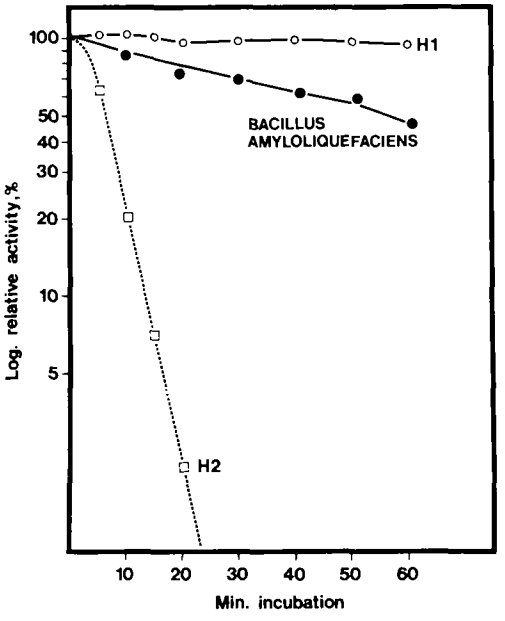

Figure 8. Time course of thermoinactivation of Bacillus hybrid β-glucanases H1 and H2 at 65 °C, pH 5.5 in comparison with the enzyme of B. amyloliquefaciens.

The purified amyloliquefaciens and H1-enzymes were dissolved at a concentration of 1 μg · ml^{-1} in 40 mM Na-acetate, pH 5.5, 10 mM $CaCl_2$ and 50 μg · ml^{-1} bovine serum albumine. The H2-enzyme preparation was dissolved at a protein concentration of 0.75 mg · ml^{-1} in an identical buffer. Samples were withdrawn periodically and assayed for residual β-glucanase activity.

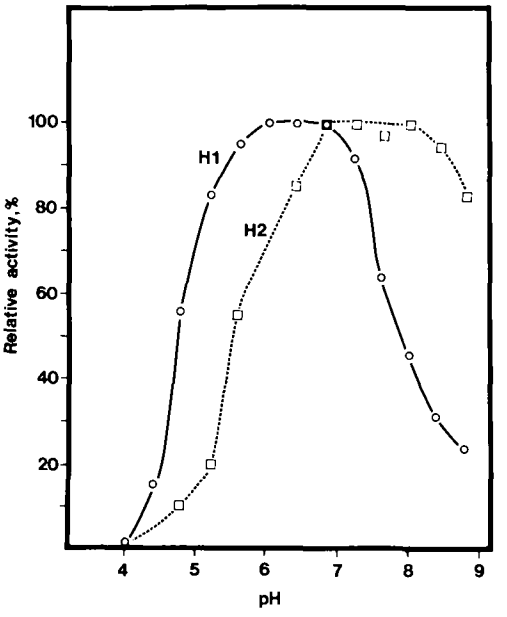

Figure 9. The pH dependance of the activity of Bacillus hybrid β-glucanases H1 and H2.

The reactions were carried out with 1-6 μg H1 β-glucanase and 7-70 μg H2 β-glucanase preparation in the following buffers: 40 mM Na-acetate, pH 3.6-5.6; 40 mM K/Na phosphate, pH 6-8 and 40 mM Tris-HCl, pH 8.4-8.8. Activity was determined with the Biocon assay using azo-barley β-glucan as substrate (19).

Coomassie blue staining band (Fig. 5). The yield of hybrid enzyme H2 was only 1% of that obtained of H1 and too low to produce a chromatographically pure preparation (Table I). The specific activity of β-glucanase H1 was determined to be 3700 μmole glucose mg^{-1} · min^{-1} which is comparable to the specific activity of β-glucanases from Bacillus IMET B376 (1330 μmole glucose mg^{-1} · min^{-1}) (6) and from B. macerans (5030 μmole glucose mg^{-1} · min^{-1}). For characterization of the bgl-H2 gene product an enriched extract with a specific activity of 10.4 μmole glucose mg^{-1} · min^{-1} was used (Table II).

3.3. Substrate specificity

Hybrid enzymes H1 and H2 degraded barley (1-3,1-4)-β-glucan as well as lichenan and the relative V_{max} values determined with both substrates did not differ significantly (Table II). The K_m values for both hybrid proteins were determined using either barley β-glucan or lichenan as substrate. The obtained values were very similar (Table II).

3.4. Kinetics of thermoinactivation of β-glucanases

Initial information about the thermostability of hybrid β-glucanases in comparison with the parental enzymes from B. amyloliquefaciens and B. macerans was obtained by measuring the time course of thermoinactivation of β-glucanase in crude extracts from E. coli cells transformed with plasmids pUC13-H1, pUC19-H2, pEG1 and pUC13-Mac encoding H1, H2, B.

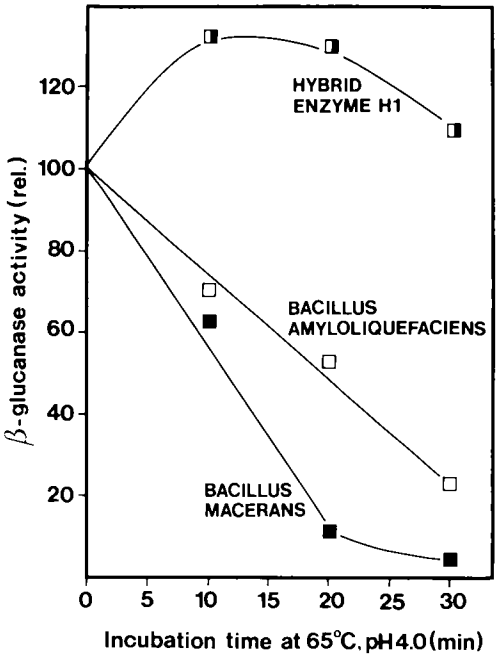

Figure 10. Activity of Bacillus hybrid β-glucanase H1 and parental enzymes in crude extracts (section 2.7) from transgenic E. coli cells after incubation for various length of time at 65 °C, pH 4.0.

Activity is expressed as per cent of activity at time 0.

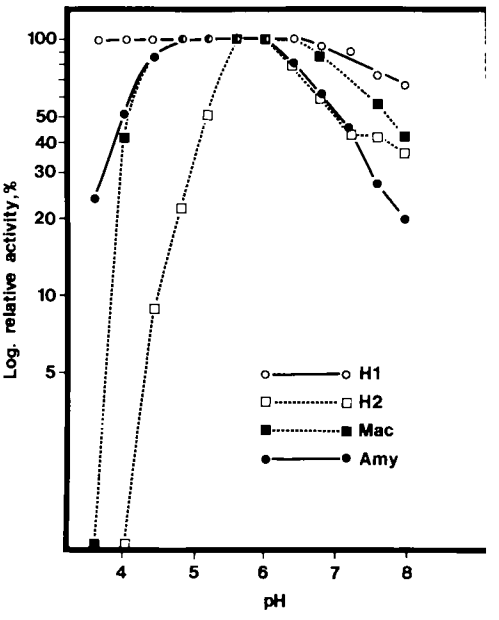

Figure 11. The pH dependence of stability of Bacillus β-glucanase at 55 °C. 2 μg of hybrid β-glucanase H1, 375 μg protein of hybrid β-glucanase H2 preparation, 2 μg of B. macerans β-glucanase or 10 μg of B. amyloliquefaciens β-glucanase were tested with 10 mM CaCl and 50 μg · ml^{-1} bovine serum albumin in 40 mM Na-acetate buffer adjusted to the indicated pH values in the range of 3.6 to 5.6 or in 40 mM K-Na phosphate buffer adjusted to the indicated pH values in the range 6.0 to 8.0. After incubation for 1 h at 55 °C the residual activity was measured with method A (section 2.7).

amyloliquefaciens and B. macerans recombinant β-glucanase, respectively. The samples (usually in the concentration range 0.3-1 mg/ml) were incubated in 10 mM $CaCl_2$, 40 mM Na-acetate, pH 6.0 at 70 °C and samples were removed periodically for determination of residual β-glucanase activity (Fig. 6). The results of this analysis reveal that the half-life of H1 β-glucanase is significantly higher (50% inactivation in 18.5 min) than half-lives of the parental enzymes from B. amyloliquefaciens (4 min) and B. macerans (9 min). The H2 β-glucanase underwent thermoinactivation with a half-life less than 2 min and is thus more heat-labile than the parental enzymes. When the analysis was carried out at 65 °C (Fig. 7) the hybrid enzyme H1 was stable for more than 30 min while the half-life of the enzyme from B. amyloliquefaciens was about 25 min and that of B. macerans intermediate between the two. Purified H1 enzyme was stable for more than 1 h when analyzed at 65 °C, pH 5.5, whereas partially purified H2 enzyme was irreversibly thermoinactivated within 20-25 min (Fig. 8). A time course for the inactivation of purified enzyme from B. amyloliquefaciens is given for comparison. Consistently, the hybrid enzyme H1 was significantly activated when tested after 5 min at 65 to 70 °C (Figs. 6 and 7).

3.5. Effect of pH on enzymatic activity and stability of hybrid β-glucanases

The pH for optimal enzymatic activity of

hybrid β-glucanase H1 was pH 5.6 to 6.6, while that for hybrid enzyme H2 was pH 7.0 to 8.0 (Fig. 9). For comparison, the pH optimum for enzymatic activity of the β-glucanases from B. amyloliquefaciens and B. macerans was pH 6.0 to 7.0 and pH 6.0 to 7.5, respectively (results not shown). Figure 9 also shows that hybrid enzyme H1 retains 50% of its activity at pH 4.8 and that H2 retains 50% of its activity at pH 5.6. The corresponding values for the parental enzymes are pH 5.2 (B. amyloliquefaciens) and pH 5.5 (B. macerans).

Another characteristic is enzyme stability as a function of pH. When the time course of thermoinactivation of the β-glucanases in crude extracts was followed at pH 4.0 and a temperature of 65 °C the hybrid enzyme H1 was stable for more than 30 min while the β-glucanase from B. amyloliquefaciens had a half-life of 20 min and that of B. macerans of only 12 min (Fig. 10). This feature was further examined for

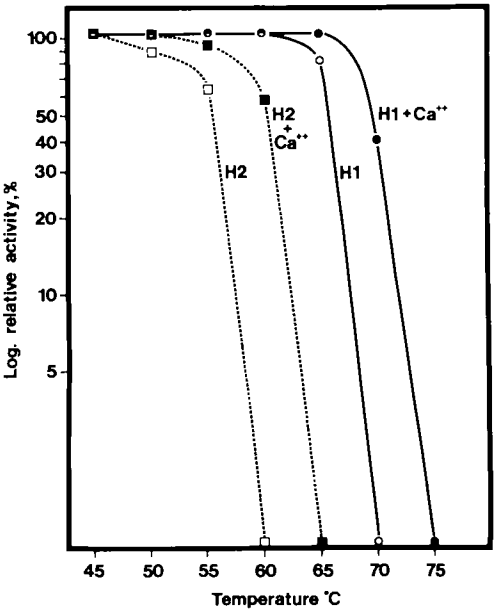

Figure 12. Improvement of thermal stability of Bacillus hybrid β-glucanases with $CaCl_2$.

0.1 µg Bacillus hybrid enzyme H1 or 750 µg hybrid enzyme H2 preparation was dissolved in 1 ml 40 mM Na-acetate buffer pH 5.5 with or without 50 mM $CaCl_2$ and supplemented with 50 $\mu g \cdot ml^{-1}$ bovine serum albumine. After incubation for 30 min at the indicated temperatures the residual activity was determined.

hybrid and parental β-glucanases by incubation at 55 °C for 1 h in the range pH 3 to 9, followed by determination of residual enzymatic activity (Fig. 11). One can see that β-glucanase H1 is stable from below pH 3.6 up to 7.0, while β-glucanase H2 has a very narrow pH range of stability between pH 5.6 to 6.0. Both parental β-glucanases are unstable below pH 4.8 and above pH 6.0 (B. amyloliquefaciens) or pH 6.4 (B. macerans).

3.6. The effect of Ca^{++} on thermostability

The effect of Ca^{++} on the stability of hybrid β-glucanases was analyzed in a 30 min assay at pH 5.5 and temperatures ranging from 45 °C to 75 °C. From the results of this analysis, shown in Figure 12, the temperature for 50% inactivation in a 30 min assay can be deduced. It is clear that Ca^{++} ions have a stabilizing effect on both hybrid enzymes. The temperatures for 50% inactivation increase about 5 °C for both hybrid β-glucanases in the presence of 10 mM Ca^{++}. The same stabilising effect of Ca^{++} ions is also found for the two parental enzymes.

4. DISCUSSION

In recent years a number of attempts have been made to construct improved versions of existing biologically active proteins to make them better suited for industrial processes and to widen their range of application. Much interest has focused on increasing the thermostability of enzymes. It has been proposed that the thermostability of enzymes may be enhanced by single amino acid substitutions that decrease the entropy of unfolding (19). Several tentative rules for increasing the thermostability of proteins have been established (1, 15, 24) but precise predictions for changes of structure to function relationships remain elusive (see ref. 26 for review).

The most thermostable (1-3,1-4)-β-glucanase known to date is produced by B. macerans (5). However, at temperatures exceeding 65 °C and at pH values of 4.5 to 5.5, which is typical for industrial mashing in a brewery, the B. macerans β-glucanase is rapidly inactivated. In the present paper we have described the construction of two

hybrid genes encoding recombinant Bacillus (1-3,1-4)-β-glucanases. These hybrid genes were constructed by reciprocal exchanges of the amino-terminal and carboxy-terminal halves of the β-glucanase encoding genes from B. amyloliquefaciens and B. macerans via a common EcoRV restriction endonuclease site located in a homologous region in the central part of the two genes. The hybrid β-glucanase genes were expressed in E. coli cells and the biochemical properties of the hybrid enzymes were analysed.

The level of β-glucanase expression from the hybrid genes in E. coli differed dramatically. Cells harbouring the H1 gene (containing 5' half of the amyloliquefaciens gene) had about 1000 fold more β-glucanase activity than cells carrying the H2 construction, but the enzyme kinetic parameters K_m and relative V_{max} for two substrates of the enzymes are similar. It is therefore most likely that this difference is caused by variation in the rate of initiation of transcription of the two hybrid genes. Possibly, the 99 nucleotides upstream from the B. macerans initiation codon in H2 constitute too short a sequence to promote efficient initiation of mRNA transcription, in contrast to the 469 nucleotides present in front of the initiation codon of H1. It cannot be excluded, however, that the H2 protein is degraded or inactivated in E. coli at a much higher rate than the H1 protein.

Compared to the parental enzymes the hybrid proteins exhibit novel biochemical properties such as different pH-optima, thermostability and differences in pH tolerance. The H1 protein is of special interest for the brewing industry since in this protein the tolerance to lower pH and a low pH optimum of enzymatic activity has been combined with a thermostability exceeding that of the B. macerans β-glucanase at high pH. The pH optimum and especially the pH tolerance has been shifted to more acidic conditions and the thermostability surpasses that of both parental enzymes over the entire tested pH range.

In vitro recombination of homologous genes giving rise to hybrid proteins retaining the biological activity of the parental molecules is not unprecedented. STREULI et al. (28) as well as WECK and coworkers (31) constructed hybrid human leukocyte interferon genes. Some of the hybrid interferons extended the host cell range for protection against Vesicular Stomatitis and encephalomyocarditis virus. Thus the AD hybrids combining portions of interferons A and D elicited significantly greater antiviral activities than either parental molecule on mouse L-929 cells, human Hela cells and primary rabbit kidney cells. Heat stability, pH stability and antigenic specificity were the same in the hybrid and parental interferon molecules.

The hybrid Bacillus β-glucanase (H1) described in this presentation is unique in combining and improving two industrially important enzyme characteristics within the same molecule. Further improvement of the hybrid β-glucanase may be accomplished by site directed or random mutagenesis in specific regions of the gene.

The superiority of the hybrid Bacillus β-glucanase H1 can be considered a case of molecular heterosis or hybrid vigour. Heterosis, the concept of superiority of heterozygotes in many genes, as recognized by G.H. SHULL in 1908 (27), forms the basis for the immensely successful breeding programs for hybrid corn and other agricultural plants. Subsequently, monohybrid heterosis or overdominance was discovered, implying that an individual heterozygous for a pair of alleles is superior to both homozygotes. The best known case in humans is the advantage provided by heterozygosity for the sickle-cell hemoglobin $Hb^s\beta$ allele in protection against the malaria parasite. The amino acid substitution of one glutamate residue by valine in the S. hemoglobin causes it to polymerize at low oxygen tension leading to severe, often fatal anemia. The hybrid hemoglobin consisting of 2 molecules HbA and 2 molecules HbS can function normally except at very low oxygen tension, when hemoglobin polymerization causes the erythrocytes to attain a sickle shape. Oligomeric hybrid glutamate dehydrogenase molecules in Neurospora have been found by FINCHAM (10) to combine properties of the homooligomeric counterparts and may turn out in some cases to be superior, i.e. display heterosis. In the hybrid enzyme described in the present report a more stable enzyme has been achieved through in vitro recombination leading to a novel polypeptide chain combining the amino-terminal half

of one glucanase with the carboxyl-terminal half of another highly homologous glucanase. Conceivably, such intragenic heterosis could also result from crossing over in merozygotes of bacteria or from meiotic crossing over in diploid or polyploid eukaryotic species. One can pose the question to what extent it plays a role in the evolution of enzyme molecules.

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