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Genetic manipulation of *Bacillus amyloliquefaciens*

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Summary

Application of modern gene technology to strain improvement of the industrially important bacterium *Bacillus amyloliquefaciens* is reported. Several different plasmid constructions carrying the α-amylase gene (*amyE*) from *B. amyloliquefaciens* were amplified in this species either extrachromosomally or intrachromosomally. The *amyE* gene cloned on a pUB110-derived high copy plasmid pKTH10 directed the highest yields both in rich laboratory medium and in crude industrial medium. The α-amylase activity, when compared with the parental strain, was enhanced up to 20-fold in the pKTH 10 transformant. This strain showed decreased activities for other exoenzymes, such as proteases and β-glucanase suggesting common limiting resources in the processing of these enzymes. Deletions were made in vitro in genes encoding neutral (*nprE*), alkaline (*aprE*) protease and β-glucanase (*bglA*). The engineered genes were cloned into the thermosensitive plasmid pE194, and the resulting plasmids were used to replace the corresponding wild type chromosomal genes in *B. amyloliquefaciens* by integration-excision at non-permissive temperature. The double mutant deficient in the major proteases (*ΔnprEΔaprE*) showed about a 2-fold further enhancement in α-amylase production in the industrial medium compared with the relevant wild type background, both when plasmid-free and when transformed with pKTH10; this strain also produced elevated levels of the chromosomally-encoded β-glucanase; pKTH10 was stably maintained both in the wild type strain and in the *ΔnprEΔaprE* mutant. We suggest that the higher yields in α-amylase and β-glucanase in the *ΔnprEΔaprE* strain are primarily due to improved access to limiting resources, and that decreased proteolytic degradation may have had a secondary role in retaining the high activity obtained.

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Bacillus amyloliquefaciens; Strain improvement; α-Amylase; Enzyme production; Deletion; Protease gene

Introduction

In the production of bacterial industrial enzymes, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* account approximately for 50% of the wordlwide enzyme market and are the major source of liquefying α-amylases, proteases and β-glucanase (Aunstrup et al., 1979; Arbige and Pitcher, 1989). *B. amyloliquefaciens* has a long history in the production of industrial enzymes (Boidin and Effront, 1917) and this species is characterized by high natural levels of production of extracellular enzymes (Ingle and Boyer, 1976). The accumulated experience in industrial processes and the high secretion capacity of *B. amyloliquefaciens* suggest this organism as an alternative host for recombinant molecules encoding bacterial exoenzymes. However, until recently, this species has escaped genetic manipulation due to the lack of basic genetic tools, such as transformation protocols. The introduction of efficient protoplast and electroporation transformation methods has enabled routine genetic manipulation of *B. amyloliquefaciens* with recombinant plasmids (Vehmaanperä, 1988, 1989).

In this paper, we report the use of *B. amyloliquefaciens* as a host for recombinant plasmids, and discuss the effect of different plasmid constructions, whether amplified extrachromosomally or intrachromosomally, on extracellular enzyme production directed by a cloned gene. The *B. amyloliquefaciens* α-amylase gene (Palva et al., 1981) was chosen as a model for assessing the productivity of different constructions; this gene has the additional advantage of containing the homologous sequences required for integration into the chromosome of *B. amyloliquefaciens*. In the course of the work, it was observed that *B. amyloliquefaciens* strains carrying multiple copies of the α-amylase gene produced decreased levels of other chromosomally-encoded exoenzymes, suggesting possible common limiting step(s) in expressing these proteins. For further enhancement of α-amylase production, we introduced deletions in the chromosomal genes coding for other major exoenzymes in *B. amyloliquefaciens*. Of the plasmid combinations tested, the α-amylase gene on an autonomously replicating high copy plasmid supported the highest yields, and the productivity was further increased by deleting the chromosomal genes for the two major extracellular proteases.

Materials and Methods

Plasmids, bacterial strains, growth media and culture conditions

The plasmids and bacterial strains used are listed in Tables 1 and 2. All ALKO strains of *B. amyloliquefaciens* are derivatives of the SB-I strain (ATCC 23843).

TABLE 1

Plasmids used

Plasmid	Antibiotic resistance markers	Relevant characteristics/ genes carried	Source/ reference
M13mp10bglA124/71	–	$bglA_{Bam}$	Hofemeister et al., 1986
pUB110	Km, Pm		Lacey, 1971
pUB110*cop*1	Km, Pm	low copy mutant	Maciag et al., 1988
pC194	Cm		Iordanescu, 1975
pE194	Em	thermosensitive replication	Iordanescu, 1976
pJH101	Ap, Tc, Cm	non-replicative in *Bacillus*	Ferrari et al., 1983
pKTH10	Km, Pm	$amyE_{Bam}$, ori_{pUB110}	Palva et al., 1981
pKTH1501	Km, Pm	$amyE_{Bam}$, ori_{pUB110}	Kallio, 1986
pSB92	Cm	$nprE_{Bam}$	Steinborn, 1988
pALK102	Em	ori_{pUB110}, ori_{pE194}	Vehmaanperä, 1988
pALK110	Em, Cm	$amyE_{Bam}$, ori_{pE194}	this study
pALK125	Em, Cm	$amyE_{Bam}$, ori_{pE194}	this study
pALK126	Em	$\Delta nprE_{Bam}$, ori_{pE194}	this study
pALK128	Em	$amyE_{Bam}$, $ori_{pUB110cop1}$	this study
pALK129	Em	$\Delta bglA_{Bam}$, ori_{pE194}	this study
pALK130	Em	$\Delta aprE_{Bam}$, ori_{pE194}	this study
pALK307	Ap	multiple cloning site	Helena Torkkeli, unpublished

Relevant abbreviations: *amy E* = α-amylase structural gene, *amp* = plasmid amplified, Ap = ampicillin, *apr E* = alkaline protease structural gene, subscript *Bam* = gene isolated from *B. amyloliquefaciens*, *bglA* = β-glucanase structural gene, Cm = chloramphenicol, Δ = deletion, Em = erythromycin, Km = kanamycin, *npr E* = neutral protease structural gene, ori = origin of replication, Pm = phleomycin, Tc = tetracycline.

The solid media consisted of LB-agar (tryptone, 10 g l^{-1}; yeast extract, 5 g l^{-1}; NaCl, 5 g l^{-1}; agar, 15 g l^{-1}) supplemented with an antibiotic, when approriate. Protease and β-glucanase activity on plates was visualized with 10 g l^{-1} skim milk and 2 g l^{-1} lichenin, respectively; the unhydrolyzed lichenin turned red after exposing the plate to Congo red (0.5 g l^{-1}). The plasmids were selected by adding kanamycin (Km) to 10 μg ml^{-1}, phleomycin (Pm) (Cayla, Toulouse, France) to 2 μg ml^{-1}, erythromycin (Em) 5 μg ml^{-1} or chloramphenicol (Cm) to 5 μg ml^{-1} (for amplified strains up to 50 μg ml^{-1}). Shake-flask cultivations for α-amylase production were performed either in rich medium consisting of dextrin (Merck 3006), 20 g l^{-1}; tryptone, 20 g l^{-1}; yeast extract, 10 g l^{-1}; NaCl, 5 g l^{-1}; 50 mM K-phosphate, pH 7.2, or in industrial HK-medium (Grunow et al., 1980) consisting of ground wheat, 20 g l^{-1}; corn starch, 20 g l^{-1}; soya flour, 15 g l^{-1}; yeast extract, 5 g l^{-1}; $(NH_4)_2HPO_4$, 10 g l^{-1}; KCl, 1.5 g l^{-1}, $MgSO_4 \cdot 7\ H_2O$, 0.5 g l^{-1}; $CaCl_2 \cdot 6H_2O$, 0.2 g l^{-1}, pH 7.0. The seed cultures were grown in liquid LB to $Klett_{60}$ 200 with the relevant antibiotic, and inoculated in the production media without antibiotic; the cultures were then shaken for 3 d at 250 rpm at 37°C.

TABLE 2

Bacterial strains used

Strain	Relevant genotype	Plasmid	Source/ reference
Bacillus amyloliquefaciens:			
P		–	ATCC23844
ALKO2100	wild type	–	Vehmaanperä, 1988
ALKO2715	*ΔnprE*	–	this study
ALKO2716	*ΔbglA*	–	this study
ALKO2717	*ΔaprE*	–	this study
ALKO2718	*ΔnprE ΔaprE*	–	this study
ALKO2729	wild type	pALK125	this study
ALKO2730	*amyE*::pALK125*amp*	–	this study
ALKO2731	wild type	pALK128	this study
ALKO2732	wild type	pKTH10	this study
ALKO2733	*ΔnprE*	pKTH10	this study
ALKO2734	*ΔbglA*	pKTH10	this study
ALKO2735	*ΔaprE*	pKTH10	this study
ALKO2736	*ΔnprE ΔaprE*	pKTH10	this study
Bacillus subtilis:			
ALKO260	wild type	–	Vehmaanperä and Korhola, 1986
Escherichia coli:			
DH5α	*sup*E44 *Δlac* U169 (ϕ80d *lac*ZΔM15) *end*A1 *rec*A1 *hsd*R17 *thi*-1 *gyr*A96 *rel*A1	–	Hanahan, 1985; Bethesda Research Laboratories, 1986
JM109	*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *Δ*(*lac-pro*AB) *thi* F'[*tra*D36 *pro*AB+ *lacIq lac*ZΔM15]	–	Yanisch-Perron et al., 1985

Relevant abbreviations: *amy E* = α-amylase structural gene, *amp* = plasmid amplified, *apr E* = alkaline protease structural gene, subscript *bglA* = β-glucanase structural gene, *Δ* = deletion, *npr E* = neutral protease structural gene.

DNA isolation and transformation

Plasmid DNA from *E. coli* and *Bacillus* was isolated according to Garger et al. (1983) and Hardy (1985), respectively. Chromosomal DNA from *Bacillus* was isolated essentially as in Marmur (1961). *E. coli* was transformed by the Hanahan method (1985). Plasmid DNA was introduced into *B. amyloliquefaciens* and *B. subtilis* by the electroporation method (Vehmaanperä, 1989); for the latter organism, also competent cells were used (Gryczan et al., 1978).

Cloning techniques

Restriction endonucleases and methylases, *Bal*31 nuclease and T4 DNA ligase were used according to supplier's instructions (Boehringer Mannheim, Mannheim, F.R.G., or New England Biolabs, Beverly, MA, U.S.A.). Electrophoretic restriction patterns of digested DNA were resolved in 0.8% agarose gels stained with ethidium bromide. For preparative purposes, the DNA fragments were isolated from low melting point agarose (FMC BioProducts, Rockland, ME, U.S.A.) gels by the freeze-thaw-phenol method (Benson, 1984).

PCR

For creating a deleted derivative of the *B. amyloliquefaciens aprE* gene, two fragments of *B. amyloliquefaciens* ATCC23844 chromosomal DNA were amplified by the PCR method. The following primers were synthesized according to the published sequence of the gene (homologous portion underlined; Vasantha et al., 1984): 1) 5′-GGTCTAGATCCGCAATTATATCATTGAC, 2) 5′-GGGATATC-GTAGACCCAATTTTTTTCAG, 3) 5′-GGCCCGGGTAGCAGGCGGAGC-CAGCA, 4) 5′-TCGAATTCTAGACGTTTCAGGACTTGGCC. The first homologous nucleotides of the primers were at 5, 361, 899 and 1760 bp, respectively; primers no. 2 and 4 were complementary to the published sequence (Vasantha et al., 1984). The target DNA was amplified by *Tth*I (New England Biolabs, Beverly, MA) or *Taq*I DNA polymerase (Cetus, Stanford, CA, U.S.A.) in 67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 1.5–2.5 mM $MgCl_2$, 10 mM β-mercaptoethanol, pH 8.8, in 25 cycles. A cycle consisted of a denaturation step of 2.5 min at 95°C, an annealing step of 1 min at 55°C and an extension step of 2.5 min at 70°C; with primers no. 1 and 2 the standard annealing step was preceded by an additional one of 2 min at 45°C.

Hybridization

Dot hybridization (Thomas, 1980) and Southern (1975) analysis were performed as described. The probes for dot hybrization were labelled with (^{32}P) as in Rigby et al. (1977), and those for Southern analysis were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, F.R.G.) according to the supplier's instructions. The bound radioactivity in dot blots were quantified by excising the filter and counting by liquid scintillation.

Enzyme assays and protein electrophoresis

α-Amylase was assayed using the Phadebas® amylase test (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions, except that the sample was diluted and assayed in 20 mM α-glycerophosphate, 5 mM $CaCl_2$, pH 5.9. The enzyme activity is presented in U ml^{-1}; 1 U catalyzes the hydrolysis of 1 μmole glucosidic linkage per min under the assay conditions. Protease activity was assayed by measuring the soluble azure dye released from 1% (w/v) hide powder

azure (Sigma, St. Louis, MO, U.S.A. or Calbiochem, La Jolla, CA, U.S.A.) after incubating for 20 min at 37°C with constant mixing. Neutral protease activity was measured by inactivating the alkaline protease in the sample with 1 mM phenylmethylsulfonyl fluoride (PMSF), and diluting and assaying the sample in 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.3. Alkaline protease activity was measured by chelating the Ca^{2+} required for neutral protease activity by diluting and assaying the sample in 50 mM Tris-HCl, 5 mM EDTA, pH 7.3. The reaction was terminated by adding 1/10 volume of 100 mM EDTA, 10 mM PMSF in 90% (v/v) methanol. Protease activity is presented in U ml^{-1}; 1 U produces soluble azure corresponding to an absorbance of 1.0 at 595 nm under the assay conditions. β-Glucanase was assayed by measuring the soluble azure dye released from 1% (w/v) azo barley glucan in 20 mM Na-acetate, pH 6.0 at 30°C, according to supplier's instructions (Biocon, Victoria, Australia). Enzyme activity is presented in U ml^{-1}; 1 U produces 1 μmol of reducing sugars equivalents per min under the assay conditions.

Membrane samples were prepared as described by Kallio et al. (1986). Proteins were analyzed by electrophoretic separation on 11% SDS-polyacrylamide gels (Laemmli, 1970). Immunoblotting with polyclonal rabbit antibodies raised against *B. amyloliquefaciens* α-amylase protein was essentially done as in Towbin et al. (1979) and Batteiger et al. (1982).

Plasmid stability estimations

These were done as described in Vehmaanperä and Korhola (1986).

Results

Plasmid constructions

To test production of α-amylase by different recombinant molecules, the *amyE* gene of *B. amyloliquefaciens* was transferred on to autonomously replicating plasmids of either low or high copy number; alternatively, it was integrated into the chromosome of *B. amyloliquefaciens* and amplified therein. The plasmid pKTH10, originally constructed by Palva et al. (1981), is based on multicopy plasmid pUB110 (copy number 40–50 per chromosome; Bron et al., 1988) and was used as the high copy plasmid in this study. A corresponding low copy plasmid was constructed by ligating the *amyE*-containing *Bgl*II-*Bam*HI fragment from pKTH1501 with *Bam*HI opened pUB110*cop*1 vector, which has a 4-fold reduction in copy number (Maciag et al., 1988), and transforming into *B. subtilis* ALKO260. A construction, which had the insert in pUB110*cop*1 in the same orientation as pKTH10, was isolated and the plasmid was designated pALK128 (Fig. 1a). Integration of *amyE* into the *B. amyloliquefaciens* chromosome on a non-replicating plasmid pJH101 failed despite several attempts. Therefore, it became necessary to separate the transformation and integration steps by taking advantage of the thermosensitive replication functions of pE194 (Iordanescu, 1976). With pE194 derivatives carrying DNA homologous to the *B. amyloliquefaciens* chromosome the transformation can be performed at permis-

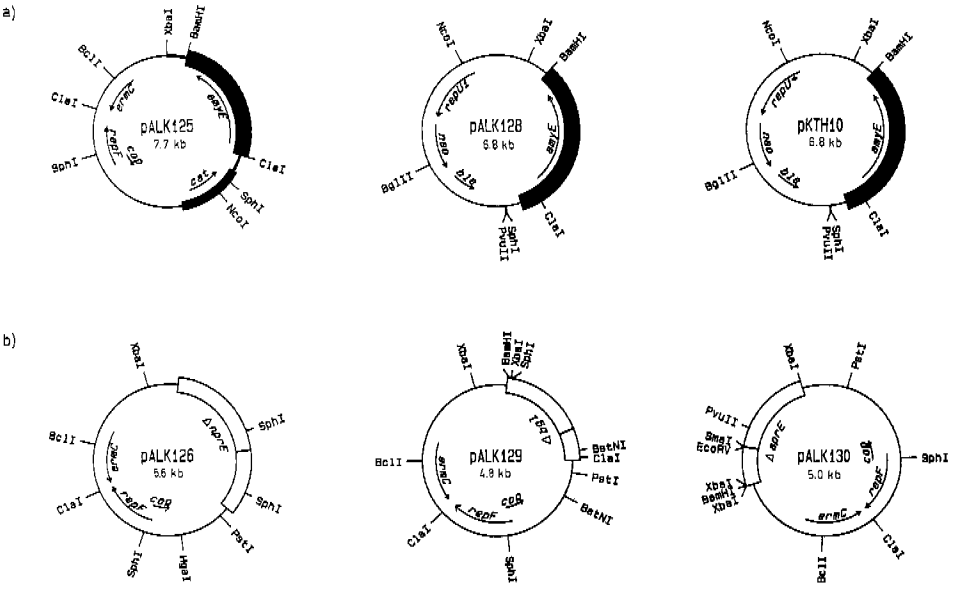

Fig. 1. Maps of the major plasmids used in this study. The plasmids used for α-amylase synthesis (a), and in constructing the deletion mutants (b) in *B. amyloliquefaciens* are depicted. The arrows show the direction of transcription; only the cloned genes, and the genes involved in antibiotic resistance or replication are shown. In (b) the division in the unfilled broad arc shows the location of the deletion. Abbreviations: *amyE*, *B. amyloliquefaciens* α-amylase gene; *ble*, phleomycin resistance determinant; *cat*, chloramphenicol transacetylase determinant; *cop*, replication control system; *ΔaprE*, deleted *B. amyloliquefaciens* alkaline protease gene; *Δbgl*, deleted *B. amyloliquefaciens* β-glucanase gene; *ΔnprE*, deleted *B. amyloliquefaciens* neutral protease gene; *ermC*, erythromycin resistance determinant; *neo*, kanamycin resistance determinant; *repF*, iniator protein determinant from pE194; *rep*U1, iniator protein determinant from pUB110*cop*1; *repU*$^{+}$, iniator protein determinant from wild type pUB110.

sive temperature and, after the plasmid has been established in the cell, the integration can be selected by cultivating the cells with antibiotic selection at non-permissive temperature. To construct a pE194-based plasmid suitable also for amplification, the pC194-derived Cm^{r} determinant (*cat*) in pJH101 was isolated on a *Hpa*II fragment, filled-in and ligated with the partially *Xba* I-cleaved and filled-in pALK102, and transformed into *B. subtilis* ALKO260. A plasmid having the *cat*-fragment inserted into the *Xba*I site nearest to the pALK102 *Eco*RI site, and with transcription of the *cat*-gene proceeding towards this *Eco*RI site, was isolated and designated pALK110 (physical map not shown). This plasmid was digested with *Xba*I and *Stu*I and the fragment carrying the pUB110 origin of replication was replaced with the *amyE*-containing *Xba*I-*Pvu*II fragment of pKTH10. The resulting recombinant plasmid was designated pALK125 (Fig. 1a).

Effect of plasmid construction on the cloned α-amylase production

The *Bam*HI site on pALK125 (Fig. 1a) was modified in vitro by *Bam*HI methylase to prevent cleavage by the endogenous *Bam*HI endonuclease

(Vehmaanperä, 1988) and the plasmid was transformed into *B. amyloliquefaciens* ALKO2100 by Em-selection at a temperature (33°C) permissive for autonomous plasmid replication. In one of the transformants, ALKO2729, the integration of pALK125 into the chromosome was selected by growing the cells in the presence of 5 μg Cm ml^{-1} at a temperature (48°C) non-permissive for plasmid replication. The culture was then challenged with gradually increasing concentrations of the antibiotic at 48°C. Clones resistant to 10, 25 and 50 μg ml^{-1} Cm were isolated, and their α-amylase production was tested in shake cultures in the dextrin - double-strength LB at 37°C. The productivity of the clones increased concomitantly with higher Cm resistance, and was approximately twice as high (about 900 U ml^{-1}) in the strains resistant to 50 μg ml^{-1} Cm as in the unamplified strain ALKO2729, and about 13 times as high as in the parental strain *B. amyloliquefaciens* ALKO2100. On gel electrophoresis, chromosomal DNA from four independently isolated clones resistant to 50 μg ml^{-1} Cm showed the expected pattern of strongly fluorescent bands against weak background bands after cleavage with restriction enzymes, verifying that the integration had taken place via the homologous *amyE* gene fragment, and that the Cm resistance was due to the amplification of the *cat*-gene moiety on pALK125 (Young 1984); the clones varied only slightly in their yields of α-amylase (details not shown). One of the strains, ALKO2730, was chosen for further studies; this strain showed about 20-fold amplification of the *amyE* gene, as estimated by the dot blot analysis.

The autonomously replicating plasmids pALK128 (low copy) and pKTH10 (high copy) were methylated in vitro at their *Bam*HI sites (Fig. 1a) and introduced in *B. amyloliquefaciens* ALKO2100, thus obtaining the strains ALKO2731 and 2732,

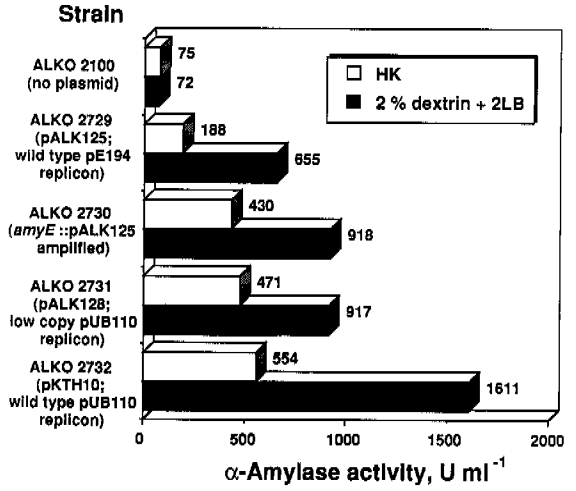

Fig. 2. α-Amylase production by the *B. amyloliquefaciens* recombinant strains in shake cultures. The strains were cultivated in the rich laboratory medium (2% dextrin + 2LB) or in the industrial medium (HK) for 3 d at 37°C.

respectively. The production of α-amylase by the strains ALKO2100, 2730, 2731 and 2732 was tested in shake cultures in the dextrin - double-strength LB and in the industrial HK-medium (Fig. 2). In both media ALKO2732 harboring the high copy plasmid pKTH10 showed the highest yields, producing about 22 (LB) and 7 (HK) times as much α-amylase activity as the parental strain ALKO2100, and about 1.8 (LB) and 1.2 (HK) times as much as ALKO2730 (pALK125 amplified) and ALKO2731 (pALK128).

Construction of deletion mutations in the chromosome of B. amyloliquefaciens

The activity of other major secreted enzymes – proteases and β-glucanase – of ALKO2732, a pKTH10 harboring strain, was reduced to about 10–25% of the level produced by the parental strain under the same growth conditions (Table 3); pUB110 did not affect the production of exoenzymes. When pKTH10 was cured, the enzyme yields were restored to parental level, indicating that the pKTH10 transformants did not carry mutations affecting exoenzyme production (details not shown). These results implied that by blocking the synthesis of other major exoenzymes the cell's metabolism could be directed in favor of α-amylase synthesis. We constructed defined deletions covering the putative promoter area and the N-terminal region of the ORF in the *B. amyloliquefaciens* genes encoding neutral protease (*nprE*) β-glucanase (*bglA*) and alkaline protease (*aprE*). An internal deletion of 705 bp in the cloned *B. amyloliquefaciens nprE* gene on pSB92 (Steinborn, 1988) was constructed by removing the 0.7 kb *Bgl*II-*Pvu*I fragment, filling in the ends generated by the restriction enzyme to make them blunt and joining by ligation. The deleted gene was transferred on a 1.9 kb filled-in *Bcl*I fragment to pE194 that had been cleaved with *Acc*I and filled-in, thus obtaining the plasmid pALK126 (Fig. 1b). This *bgl*A gene of *B. amyloliquefaciens* on M13mp10 bglA124/71 (Hofemeister et al., 1986) was cleaved at the *Bam*HI site at 416 bp, digested with *Bal*31, cut with *Eco*RV at 858 bp and joined by ligation, thus creating a deletion of about 500 bp. The deleted *bgl*A gene was transferred on a 1.1 kb *Eco*RI–*Pvu*I fragment (*Pvu*I site filled-in) into *Eco*RI-*Hin*cII-cleaved *E. coli* plasmid pALK307 (a derivative of pIBI76 of International Biotechnologies, New Haven, CT, U.S.A.; Helena Torkkeli, unpublished), thus resulting in the plasmid pALK127 (physical map not shown). pALK127 was digested with *Eco*RI, filled-in, and inserted into pE194 that had been cleaved with *Acc*I and filled-in, producing the plasmid pALK129 (Fig. 1b). Due to an unintended partial duplication of the pALK307 multiple cloning site, this plasmid carries *Bam*HI and *Xba*I sites at the distal end of the *bgl*A gene. A deleted derivative of the *B. amyloliquefaciens aprE* gene was constructed by joining in the original orientation the PCR amplified 5-to-361 and 899-to-1760 fragments (Vasantha et al., 1984), thus generating a deletion of 537 bp. The deleted gene was inserted at the *Xba*I site of pE194, and the resulting plasmid was designated pALK130 (Fig. 1b).

The approach for transferring the deleted genes into the chromosome of *B. amyloliquefaciens* was to integrate the thermosensitive plasmid by the Campbell-type recombination (Ferrari et al., 1983) at non-permissive temperature by maintaining

antibiotic selection for the plasmid, and after establishing the integration, allowing the curing of the plasmid by cultivating the cells at 48°C in antibiotic-free medium: when the integrated plasmid is excised on the opposite side of the deletion, the plasmid-carried DNA sequence is retained in the chromosome and the wild-type

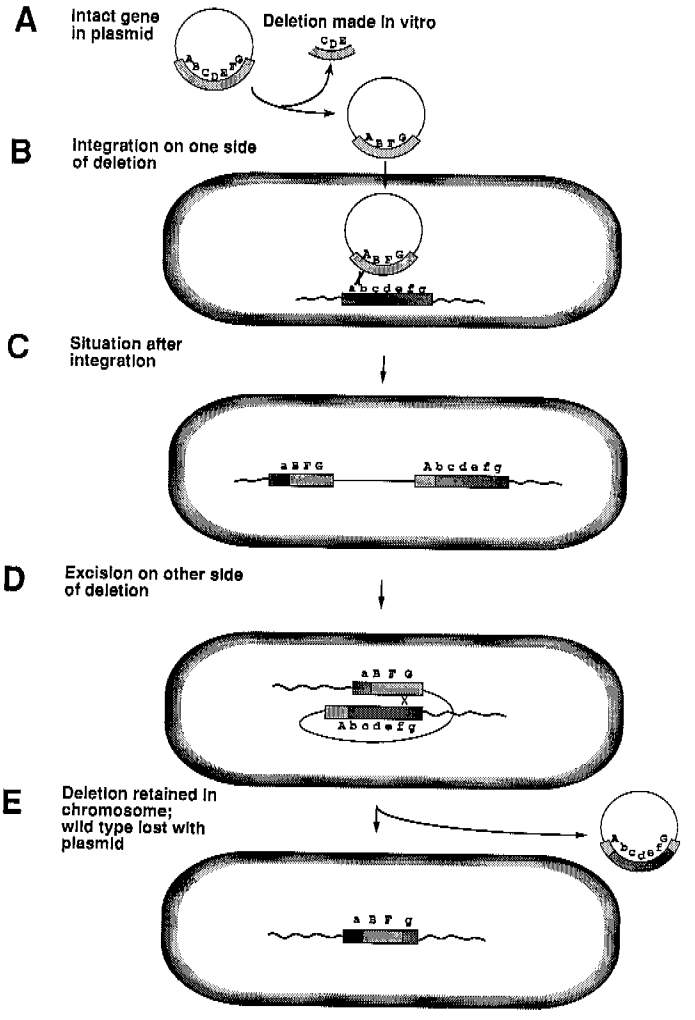

Fig. 3. Schematic presentation of the approach for transferring the deleted genes into the *B. amyloliquefaciens* chromosome. The deleted gene is cloned into the thermosensitive replicon pE194 (A), and the resulting plasmid is transformed into the cell at permissive temperature (33°C) (B). The cells with an integrated copy of the plasmid are selected by maintaining antibiotic selection at restrictive temperature (48°C) (B to C). The excision of the integrated copy is allowed by cultivating the cells at restrictive temperature (48°C) without antibiotic (D to E). Upper- and lowercase letters indicate complementary of the DNA sequence, the grey broad arcs and rectangles the gene of interest, the thin line the non-homologous portion of the plasmid and the thick wavy line the non-homologous portion of the chromosome.

copy is lost with the plasmid (Fig. 3). Plasmids pALK126, pALK129 and pALK130 (the latter two methylated at their *Bam*HI sites) were transformed in *B. amyloliquefaciens* ALKO2100 at permissive temperature (33°C), and were integrated into the chromosome by growing in Em-medium at 48°C for 3–4 cycles of growth. The strains were streaked on Em-plates at 37°C, and were then grown for 3–4 cycles without antibiotic at 48°C before plating on screening plates. Clones carrying *ΔnprE* and *ΔbglA* mutations could be detected on plates by the absence of haloes on skim milk and lichenin plates, respectively. However, *B. subtilis* clones carrying a *ΔaprE* mutation are known to produce a normal halo on skim milk plates (Yang et al., 1984), and a different approach was taken to screen for these mutants in *B. amyloliquefaciens*. Clones were grown in LB in microtiter wells, and alkaline protease activity was assayed from the overnight culture medium with succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrate (DelMar et al., 1979). Clones remaining pale yellow after the assay were regarded as potential mutants. The frequency of $NprE^-$ mutants after the integration-excision was well above 1%, whereas $BglA^-$ and $AprE^-$ mutants were found at a frequency of about 0.1%. The Em sensitivity of the mutants was verified, and the strains obtained were designated ALKO2715 (*ΔnprE*) ALKO2716 (*ΔbglA*) and ALKO2717 (*ΔaprE*). ALKO2715 was further mutagenized by replacing the chromosomal *aprE* gene with the deleted derivative on pALK130 as described above. In this case, the *ΔaprE* mutants could be screened on plates, since ALKO2715 developed a small halo on skim milk after 24 h incubation, whereas the double mutant *ΔnprEΔaprE* produced no detectable halo; again, the frequency of mutants was about 0.1%. The double mutant strain was designated ALKO2718.

Characterization of the deletion mutants

The enzyme activity pattern of the *B. amyloliquefaciens* mutant strains after cultivating in the HK-medium was as expected (Table 3): the protease negative mutants ALKO2715 and ALKO2717 showed in the hide powder azure assay only residual or no activity of the neutral and alkaline protease, respectively, while the double mutant ALKO2718 produced in this assay no detectable protease activity. However, supernatants of the *ΔaprE* strain ALKO2717 still showed about half of the activity of the wild strain towards the synthetic peptide succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, presumably due to some minor protease(s). This activity was inhibited by 1 mM PMSF, but unaffected by 10 mM EDTA. The β-glucanase negative mutant ALKO2716 produced only residual activity in the azobarley glucan assay (Table 3). The chromosomal DNA of the parental strain and the mutants was isolated and subjected to Southern analysis with the relevant gene as a probe (Fig. 4). When compared to the parental strain, all the mutants showed probe-specific fragments with deletions of expected size. As a control, the chromosomal DNA of ALKO2715 was probed with labelled pE194, but no hybridization was observed, indicating that no remnants of the pE194 moiety in pALK126 were retained in the chromosome.

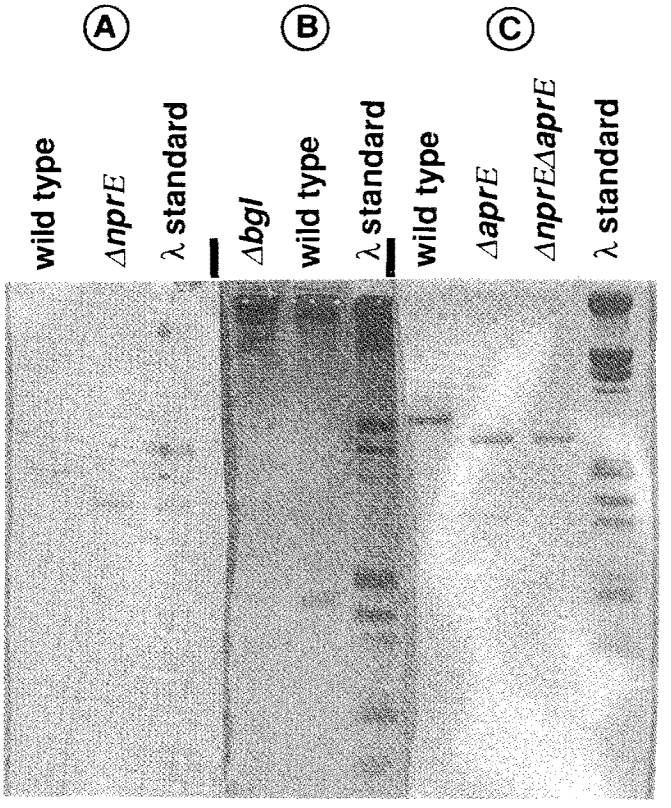

Fig. 4. Southern blots of chromosomal DNA from the wild type *B. amyloliquefaciens* (ALKO2100) and from the strains carrying deletions in genes encoding neutral protease (ALKO2715; *ΔnprE*), β-glucanase (ALKO2716; *ΔbglA*) or alkaline protease (ALKO2717; *ΔaprE*), or in both genes encoding the major proteases (ALKO2718; *ΔnprE ΔaprE*). The cloned genes of *ΔnprE* in pALK126 (A), *ΔbglA* in pALK129 (B) and *ΔaprE* in pALK130 (C) were used as a probe. The chromosomal DNA in (A) and (C) was cut with *Hin*dIII and in (B) with *Nco*I. The λ standard was cut with *Eco*RI and *Hin*dIII, and was hybridized with labelled λ DNA.

α-Amylase production by the deletion mutants

pKTH10, the construction resulting in the highest α-amylase yields in a strain with the wild type background (Fig. 2) was transformed in ALKO2715, 2716, 2717 and 2718, thus providing strains ALKO2733, 2734, 2735 and 2736, respectively. The plasmid-free strains and the pKTH10 transformants were cultivated in shake cultures in the industrial HK-medium and compared to the parental strain (Fig. 5). Both plasmid-free and pKTH10-containing protease negative strains showed superior activities in α-amylase compared to the respective protease positive strains. The double deficient strains ALKO2718 and ALKO2736 produced about twice the activity of the corresponding wild type strains ALKO2100 and ALKO2718 and the α-amylase production was enhanced also in strains carrying a single *ΔnprE* or

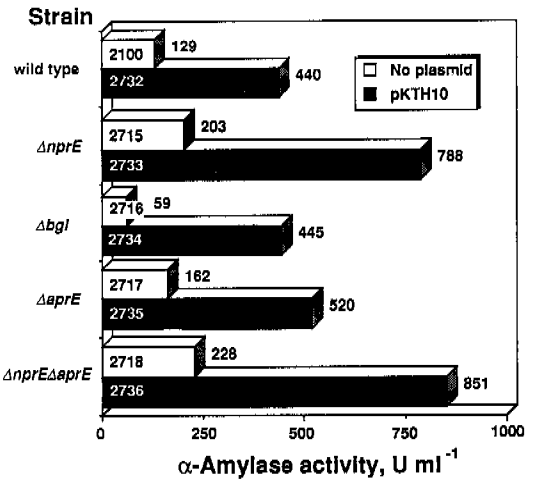

Fig. 5. α-Amylase production by the *B. amyloliquefaciens* strains carrying deletions in genes encoding neutral protease (*ΔnprE*), β-glucanase (*ΔbglA*) and alkaline protease (*ΔaprE*). The numbers within the bars indicate the relevant ALKO strain number. The strains were cultivated in the industrial medium (HK) for 3 d at 37°C.

ΔaprE mutation (Fig. 5). Also the β-glucanase encoded by the chromosomal gene showed an about 2-fold increase in production in the protease negative mutants. Again, the highest yields were obtained with the double mutant (8.4 U ml^{-1} with ALKO2736 vs 4.5 U ml^{-1} I with ALKO2732). The increase in α-amylase activity was much less pronounced, only 20–30% higher, when the protease negative mutants were grown in the dextrin - double-strength LB medium (data not shown). On the other hand, the *ΔbglA* strains did not show elevated α-amylase activities (Fig. 5). ALKO2732 and 2736 showed similar maximal viable counts (plated after overnight growth in the rich medium), suggesting that the superior productivity of ALKO2736 was not due to a higher cell number. With membranes from the strains ALKO2100, ALKO2729, ALKO2730, ALKO2731, ALKO2732 and ALKO2736 (grown 24 h in the HK-medium), immunoblots containing the same amount of protein, or material from same amount of cells showed a gradual increase in the intensity of the α-amylase precursor band, parallel to the increased α-amylase activities produced by the strains (Figs. 2 and 5; data not shown). pKTH10 was both structurally and segregationally stable in the strains ALKO2732 and ALKO2736 (< 0.5% cells cured after cultivating about 50 generations without antibiotic selection).

Comparison by SDS-PAGE electrophoresis and by immunoblotting of the proteins secreted into the culture media by the wild type and the protease negative mutant strains indicated no significant difference in proteolytic degradation of α-amylase. Occasionally when supernatant from a shake culture with unexpected low α-amylase activity was analysed in SDS-PAGE electrophoresis a weakened α-amylase band was observed together with a new band with a mobility corresponding to about a 42 kDa protein, which thus may represent an inactive degradation product of α-amylase. However, polyclonal rabbit antibodies raised against *B.*

TABLE 3

Enzyme production by the mutants carrying deletions in genes encoding neutral protease, alkaline protease, and β-glucanase [a]

Strain	Genotype	Neutral protease activity, U ml^{-1} [b]	Alkaline protease activity, U ml^{-1} [b]	β-Glucanase activity, U ml^{-1} [d]
ALKO2100	wt	88.0	38.2	16.8
ALKO2715	*ΔnprE*	1.6	88.3	
ALKO2716	*ΔbglA*			3.2
ALKO2717	*ΔaprE*	81.4	nd [c]	
ALKO2718	*ΔnprE ΔaprE*	nd [c]	nd [c]	
ALKO2732	wt/pKTH10	21.4	2.1	3.9

[a] Strains were grown in shake cultures in the industrial HK-medium for 3 d at 37 °C; [b] Protease activity was measured with the hide powder azure assay, using 1 mM PMSF to inactivate alkaline protease when neutral protease was assayed, and 5 mM EDTA to inactivate neutral protease when alkaline protease was assayed; [c] not detectable; and [d] β-glucanase activity was measured with the azo barley glucan assay.

amyloliquefaciens α-amylase recognized only the native α-amylase protein in immunoblotting (details not shown).

Discussion

In this paper, we demonstrate the application of recombinant DNA techniques in strain development of a traditional industrial microbe, *B. amyloliquefaciens*. Recombinant molecules were established as extrachromosomal elements as well as integrated and amplified intrachromosomally. We were also able to introduce in vitro derived deletions into the chromosome of *B. amyloliquefaciens* thus creating the first defined mutations reported for this organism. Using the α-amylase gene as a model, we tested several vector constructions, and obtained the highest yields when the gene was carried on a high copy plasmid pKTH10 (Fig. 2). The stability of pKTH10 was high, in agreement with the previous results obtained in *B. subtilis* (Vehmaanperä and Korhola, 1986); no deletions in pKTH10 were observed at any stage, although that this plasmid contains about 2 kb DNA homologous to the chromosome. In the case of the amplified system, the residual replication of the pE194 moiety of the pALK125 at the production temperature of 37°C may unfavorably interfere with the α-amylase synthesis or with the cell's physiology more generally (Noirot et al., 1987), and may explain why as high an increase in enzyme production was not obtained as reported for the amplified strains of *B. subtilis* (Kallio et al., 1987). However, the use of a thermosensitive plasmid was our only choice for establishing integration, since no transformants were obtained with a non-replicating recombinant plasmid. Integration requires activity of the cell's recombination machinery (Niaudet et al., 1982), which, unlike in competent cells (de Vos and Venema, 1982), does not seem to be induced in protoplasts or cells prepared for electroporation.

The low probability of both successful DNA entry and integration shortly thereafter is likely to reduce the frequency of transformation with non-replicating plasmids to below detection level in these cells.

The increase in α-amylase production with the pKTH10 transformant ALKO2732 was, depending on the medium used, about 10- to 20-fold compared to the unmanipulated parental strain (Fig. 2). The other secreted enzymes tested – proteases and β-glucanase – were suppressed in ALKO2732 (Table 3), suggesting that all these proteins compete for some limiting resources. The corresponding genes were deleted from the chromosome in order to test if this would enhance α-amylase synthesis. The deletions covered the putative promoter and N-terminal areas of the genes, and were intended to block both the transcription and the translation of the gene. The approach used in this study for creating deletions – exchange of the deleted gene derivative with the wild type copy by integration-excision – has the advantage that no foreign DNA is retained in the mutant chromosome and multiple deletions in different genes can successively be introduced by the same procedure. The frequency of mutants was about 10-fold higher when using pALK126, compared with pALK129 and pALK130. This probably reflects the length of the fragment upstream of the deletion, which in pALK126 was about 750 bp compared with 300–400 bp in the other two. The *ΔnprEΔaprE* strain ALKO2718 did not produce any detectable halo on milk plates nor did it produce any activity in the hide powder azure assay (Table 3) in agreement with the results obtained in *B. subtilis* strains with similar deficiency (Yang et al., 1984). The residual proteolytic activity towards the synthetic peptide succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide has characteristics of *B. subtilis* bacillopeptidase F (Sloma et al., 1990; Alan P. Sloma, personal communication), but further studies are needed to verify this tentative identification. The residual β-glucanase activity in the *ΔbglA* strain ALKO2716 (Table 3) may be due to other proteins with glucan-hydrolysing activity; several genes encoding for such polypeptides have been mapped in *B. subtilis* (Borriss et al., 1986).

The mutants with deletions in genes for the major proteases showed superior α-amylase production in the industrial medium, both when plasmid-free and when transformed with pKTH10 (Fig. 5), but in the rich laboratory medium the difference between the wild type and the mutant strains was less significant; this medium has high contents of free amino acids and it does not contain β-glucan, resulting in low expression of proteases and β-glucanase in strains carrying wild type alleles (Ingle and Boyer, 1976). The hypothetical limiting resources for the enzymes may be at any level from transcription to translocation and processing, and since no strains with inactive genes encoding intracellular proteins were tested, there is no evidence that they are involved only with proteins to be exported. Attractive candidates as such limiting factors are, e.g., the DegQ and DegU (formerly SacQ and SacU) proteins, which are positive regulators enhancing the transcription of genes for α-amylase, proteases, β-glucanase and other degradative enzymes, including intracellular ones; their target areas map about 100–160 bp upstream of the transcription start point (Henner et al., 1988). These proteins have been studied in *B. subtilis*, but the *degQ* counterpart has also been cloned from *B. amyloliquefaciens*

(Tomioka et al., 1985) and it seems safe to assume that the *degS-degU* operon exists also in this organism (Msadek et al., 1990). The genes encoding neutral protease, alkaline protease and β-glucanase are highly homologous between *B. subtilis* and *B. amyloliquefaciens*, including the upstream promoter area (Henner et al., 1988; Hofemeister et al., 1986; Murphy et al., 1984; Stahl and Ferrari, 1984; Vasantha et al., 1984; Yang et al., 1984). Of these genes, the target area for DegQ and DegU proteins has been mapped by deletion analysis in the *B. subtilis aprE* gene and is located between -141 and -164. This area is conserved also in *B. amyloliquefaciens* (Ferrari et al., 1988; Henner et al., 1988; Vasantha et al.. 1984) and was deleted in the *ΔaprE* strains used in this study. However, the determined target areas of *aprE* and *sacB* do not share any striking similarity and in comparison with the upstream sequences of the other target genes revealed no obvious consensus sequence (Henner et al., 1988). The deletions in the *ΔnprE* and *ΔbglA* strains constructed in this study extend, respectively, about 250 bp and 50–150 bp upstream the translation start codon (the end point of the deletion in the *ΔbglA* construction is unknown because of the *Bal*31 digestion). Neither the transcription start points, nor the DegQ and DegU target areas of these genes have been located, but one could speculate that the target area has been deleted in the *ΔnprE* strain and not in the *ΔbglA* strain, and this would explain the enhancement of α-amylase production in the *ΔnprE* background, and the unaffected phenotype with the *ΔbglA* background; the deletion of the target area in the *ΔaprE* strains also coincides with higher productivity (Fig. 5). On the other hand, the α-amylase gene from *B. amyloliquefaciens* shares no significant homology with that from *B. subtilis* (Takkinen et al., 1983; Yang et al., 1983), and there is no evidence that the former gene is subject to DegQ-DegU mediated regulation; indeed, the chromosomally located *degU*(Hy) mutations failed to increase α-amylase production encoded by the plasmid-carried *B. amyloliquefaciens* gene in *B. subtilis* (Sibakov et al., 1983). Until further information about the role, if any, of the DegQ and DegU in *B. amyloliquefaciens* α-amylase regulation accumulates, their relevance to the enhancement of α-amylase production in the deletion mutants described in this paper remains uncertain.

The deletions in the genes encoding proteases, particularly neutral protease, increased the α-amylase production most significantly, whereas deletion in the β-glucanase gene had no effect (Fig. 5). Neutral protease is one of the major proteins secreted by *B. amyloliquefaciens*, accompanied by the α-amylase and alkaline protease, as indicated, e.g., by electrophoretic examination (Manabe et al., 1985; Vasantha et al., 1984; our unpublished data), and therefore it is not suprising that inactivation of the protease genes makes the greatest contribution to the enhancement of α-amylase production. On the other hand, exoenzymes serve the cell by converting organic polymers in crude media, such as in the industrial HK-medium, to easily metabolizable compounds, and lack of such activity may result in limitation of nutrients and subsequently in lower yields in enzyme production. In the HK-medium β-glucanase may be needed for full exploitation of the nutrients, whereas activity provided by the two major proteases may be expendable (see also below). However, since it is the major proteases which are absent in the overproducing strains, one cannot exclude the possibility that in these strains less

α-amylase is degraded than in the wild type strain, rather than that more is produced. α-Amylase is reported to be resistant to proteases in its native conformation, but is degraded, if stabilizing agents, particularly Ca^{2+}, are removed (Hagihara et al., 1956; Stein and Fischer, 1958). The HK-medium is supplemented with $CaCl_2$, and the partly decomposed starch in the medium also serves as a stabilizer (Hagihara et al., 1956). Although we cannot rule out occasional proteolytic degradation of the α-amylase protein, we did not find any supporting evidence for this in immunological analysis of the supernatants of the wild type and the protease deficient strains. Also, immunoblotting of membranes prepared from the wild type strain ALKO2732 and the protease deficient strain ALKO2736 showed clear monopolization of the secretion machinery by the α-amylase precursor in the latter strain (see Results). Furthermore, lack of degradation by proteases does not explain the evident suppression of other exoenzymes in strains transformed with pKTH10. Therefore, we suggest that the primary reason for the enhancement of the α-amylase (and β-glucanase) production in the strains with the deleted protease genes is improved access to some limiting resources, although the lack of protease activity may in some cases help in retaining the high activity obtained.

The results obtained in this study can be used in designing strategies for constructing other *B. amyloliquefaciens* strains for enzyme production. The defined mutations constructed may be transferred to other strains of *B. amyloliquefaciens* by generalized transduction and the deletions could be used as landmarks in constructing a genetic map for this organism (Abalakina et al., 1990). These strains may also be useful in purification of proteins, and the double protease deficient strain, ALKO2718, may further be manipulated for production of heterologous proteins susceptible to protease degradation. A similar approach as used in this study can be used for creating deletions in other *B. amyloliquefaciens* genes, the activities of which are not desired and the sequences of which are known; one such candidate is the gene encoding the *Bam*HI restriction endonuclease (Brooks et al., 1989).

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