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Increased Production of Extracellular Enzymes by the Synergistic Effect of Genes Introduced into *Bacillus subtilis* by Stepwise Transformation

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The *amyR3, amyS, papS1, tmr,* and *papM118* mutations each stimulate α-amylase production two- to sevenfold above the level in a wild-type strain of *Bacillus subtilis.* A strain which presumably has all five of these mutations produced 250-fold more α-amylase.

The production of extracellular α-amylase in bacilli is regulated by a number of genes. The most significant of these appear to be *amyR, papM,* and *tmr.* The *amyR* gene is a specific regulator of α-amylase that can be linked to its structural gene (*amyE*) by transformation (5). The *papM* gene regulates the production of α-amylase and protease as well as a number of other pleiotropic properties (1, 6). The *tmr* gene encodes resistance to tunicamycin. This mutant also produces high levels of extracellular α-amylase (3). *Bacillus subtilis* Marburg 6160 carries *amyR1* (5), and *B. subtilis* var. *amylosaccharitiçus* carries *amyR3* (7). In the absence of other regulatory genes, *amyR1* permits production of 10 U of α-amylase activity per ml, and *amyR3* permits production of 50 U/ml. It is possible to introduce genes that are also involved in the production of α-amylase from closely related bacilli into *B. subtilis* by deoxyribonucleic acid (DNA)-mediated transformation. Frequently, this procedure results in the improvement of enzyme production.

In the experiments to be described in this communication, DNA isolated from *B. subtilis* var. *amylosaccharitiçus* by the method of Saito and Miura (2) was used to transform the recipient strain of *B. subtilis* Marburg 6160 that usually produces 10 U of α-amylase per ml. Transformation was performed by the method of Yoshikawa (8), and primary selection was generally made for an auxotrophic requirement or an antibiotic marker. Primary transformants were then screened for increased protease activity by incorporation of 1% casein in the selective media. It was reasoned that the industrial strain of *B. subtilis* var. *amylosaccharitiçus* that produces 120 to 150 U of extracellular α-amylase per ml might contain more than one gene, and that incorporation of increasing numbers of related genes into a strain could increase α-amylase production proportionally. The present report not only proved this assumption but also established that the effect of multiple genes is not additive, but of a synergistic nature. For example, one of the transformants (SP38) obtained by transforming *B. subtilis* Marburg 6160 with DNA from *B. subtilis* var. *amylosaccharitiçus* produced 220 to 250 U of α-amylase per ml. This is twofold greater than the amount produced by the donor strain. Genetic analysis revealed that strain SP38 had acquired the *amyR3* gene as well as two new genes from *B. subtilis* var. *amylosaccharitiçus: papS1* and *amyS1.* The gene is denoted by the function and the strain. Thus *papS1* and *amyS1* are the genes encoding the hyperproduction of α-amylase and protease, and the regulator of α-amylase, respectively, from *B. subtilis* var. *amylosaccharitiçus.* The *papS* gene stimulates the production of α-amylase two- to threefold further, with a simultaneous 5- to 10-fold stimulation of protease production, than the *papM* gene does. However, a strain carrying the *papS* gene does not display the pleiotropic characteristics of the *papM* mutant. Genetic analyses also revealed that these genes can be separated by DNA-mediated transformation. The details of this study will be reported elsewhere (Y. Yoneda, manuscript in preparation). Surprisingly, a gene that stimulates only α-amylase production twofold was found in strain SP38, although such a gene had not been observed in either the donor strain or the recipient strain (*amyS1*). Table 1 shows the effect of these genes on the production of α-amylase and protease in *B. subtilis.*

Because strain SP38 has three genes (*amyR3, papS1,* and *amyS1*) that contribute to α-amylase production and act synergistically, experiments were carried out to determine whether or not the *tmr* and *papM118* mutations would further

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stimulate production in this strain. Therefore, the *tmr* gene and *papM118* gene were introduced stepwise by transformation. The overall approach is shown in Fig. 1. The *tmr* gene of strain B7 was transferred to strain SP38. One of the tunicamycin-resistant transformants selected (TM23) produced sixfold more α-amylase than strain SP38, indicating a high probability that it contained four genes (*amyR3, papS1, amyS1,* and *tmr*). Other *tmr* transformants also showed increased production (five- to sevenfold; Table 1). Next, the *papM118* gene of strain YN118 was transferred to strain TM23. Transformants that showed further increased productivity of protease were selected. If a strain contains *papS1* and *papM118* genes at the same time, this results in a synergistic effect on protease production. One of the transformants with high protease productivity (PP13) produced twice as much α-amylase as strain TM23. Strain PP13 may differ from strain 6160 in five genes: *amyR3, papS1, amyS1, tmr,* and *papM118*. The level of α-amylase production in strain PP13 is about 250-fold higher than in the original strain (6160) and 20-fold higher than in *B. subtilis* var. *amylosacchariticus*. The extent of synergy is summarized in Table 2. Figure 2 shows the extracellular proteins on a sodium dodecyl sulfate-polyacrylamide gel. Strains TM23 and PP13 have a prominent α-amylase band (Fig. 2, band A).

TABLE 1. *Stimulating effect of various genes*

Gene	Fold stimulation of production[a]	
	α-Amylase	Protease
amyR2[b]	4–5	1
amyR3[b]	4–5	1
amyS	2–3	1
papM118	2–3	10–20
papS1	2–3	5–10
tmr	5–7	1

[a] The original strain is *B. subtilis* Marburg 6160 (*amyR1*). Its activity is designated as 1. The fold stimulation is the extent of increase with each gene.

[b] The *amyR2* and *amyR3* genes are derived from *B. natto* IAM1212 and *B. subtilis* var. *amylosacchariticus*, respectively. They are allelic to *amyR1* gene.

Increased production of extracellular protease can be obtained by similar methods. Uehara et al. (4) reported a gene, *nprR*^{h}, from *Bacillus natto* IAM1212 which stimulates extracellular neutral protease production. When the gene is transferred to strain 6160, the transformant (NP58) produces 10- to 20-fold more protease than strain 6160. The *papM118* mutation was introduced into strain NP58 using high α-amylase production (as indicated by the presence of an increased halo around the transformants on BY medium containing 1% soluble starch) as the selection technique. One transformant, YY154, could produce fivefold more protease than strain NP58 (Fig. 1). Other transformants also showed increased production (three to fivefold). Strain YY154 may have both the *nprR*^{h} and *papM118* genes. Because the *papM118* mutation prevents competence, it was not possible to introduce *papS1* into strain YY154. However, *papS1* has no effect on competence development. Therefore, it should be possible to construct a strain containing *nprR*^{h}, *papM118,* and *papS1*. A strain could be made by transformation that would contain both *nprR*^{h} and *papS1*. Then the *papM118* mutation could be introduced into the strain. This strain would then contain the three genes (*nprR*^{h}, *papS1,* and *papM118*), and it is predicted that it would produce fivefold more protease than strain YY154.

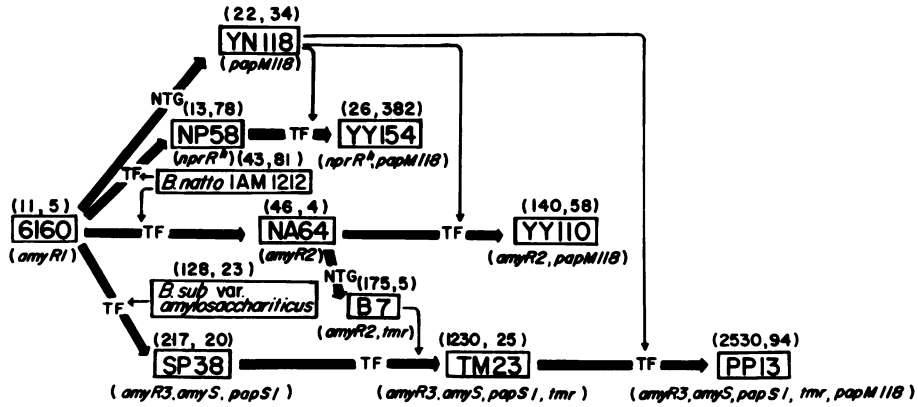

FIG. 1. *Strategy of construction of the strains. The numerals above each strain represent α-amylase and protease production (U/ml), respectively, in BY medium at 30°C for 30 h. The genetic symbols below the strain represent genes participating in production of α-amylase, protease, or both. The thick arrows indicate the direction of stepwise construction of the next strain. NTG, By N-methyl-N'-nitro-N-nitrosoguanidine treatment; TF, by transformation with DNA from the strain indicated by the thin arrow.*

TABLE 2. *Effect of genes on α-amylase production*

Strain	Gene							α-Amylase production (U/ml)	Origin (reference)
	amyR1	*amyR2*	*amyR3*	*papS1*	*amyS*	*tmr*	*papM118*		
6160	x							11.4	(5)
YN118	x						x	22.3	(6)
NA64		x						46.0	(5)
YY110		x					x	140.0	(6)
B7		x				x		175.0	(3)
SP38			x	x	x			209.0	*B. subtilis* var. *amylosaccharitiicus* $\xrightarrow{tf}$ 6160 (Y. Yoneda, in preparation)
TM23			x	x	x	x		1,475.0	B7 $\xrightarrow{tf}$ SP38
PP13			x	x	x	x	x	2,532.0	YN118 $\xrightarrow{tf}$ TM23

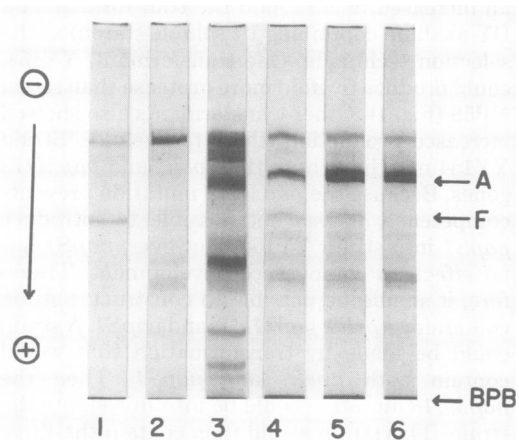

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracellular proteins produced by strains 6160 (1), YN118 (2), B. subtilis var. amylosaccharitiicus (3), SP38 (4), TM23 (5), and PP13 (6). Each strain was cultured in BY medium at 30°C for 30 h. Cells were removed by centrifugation. The supernatant was boiled with 1% 2-mercaptoethanol and 1% sodium dodecyl sulfate for 10 min and applied to the gel with bromophenol blue (BPB) as an indicator. Band A corresponds to α-amylase, and band F corresponds to flagelline monomer, which cannot be seen in the strain harboring papM118.*

The results reported in this paper demonstrate that strain improvement in the production of extracellular enzymes can be achieved by the synergistic effect of many genes. At present, not all the genes have been verified by genetic analyses. This approach to strain improvement involves the introduction, in a random fashion, of genes from strains found in nature that have a higher capacity for production. This strategy requires the use of genetically related strains. The use of genes that encode resistance to antibiotics which influence the cell wall appear to be particularly useful. In an alternative approach, one can develop vectors for cloning these genes and introducing them in a more purified form into the mother cell. This procedure does not require chromosomal homology, and therefore genes can be used from diverse sources. Hopefully, one could combine both methods to obtain markedly improved strains.

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