Cloning of a Gene Cluster of *aroB*, *aroE* and *aroL* for Aromatic Amino Acid Biosynthesis in *Brevibacterium lactofermentum*, a Glutamic Acid-producing Bacterium

Kazuhiko MATSUI, Kiyoshi MIWA and Konosuke SANO

Central Research Laboratories, Ajinomoto Co., Ltd.,
Kawasaki-ku, Kawasaki 210, Japan

Received September 10, 1987

The *aroL* gene, encoding shikimate kinase of *Brevibacterium lactofermentum*, a coryneform glutamic acid-producing bacterium, was cloned. Recombinant plasmids containing the *aroL* gene caused elevated levels of shikimate kinase synthesis in *B. lactofermentum*. It was found that in addition to the *aroL* gene, the *aroB* and *aroE* genes, encoding dehydroquinate synthase and shikimate dehydrogenase, respectively, also existed on these recombinant plasmids, in complementation tests with various *Escherichia coli* and *B. lactofermentum* aromatic amino acid auxotrophs. The *aroL*, *aroB* and *aroE* genes of *B. lactofermentum* are located closely on the cloned DNA fragment, in that order. It was shown that at least these three *aro* genes form a cluster on the chromosome of *B. lactofermentum*.

Brevibacterium lactofermentum is an industrially important Gram-positive bacterium used for the production of various amino acids. We have focused our attention on the aromatic amino acid biosynthesis of this microorganism, because phenylalanine is important as a component of "Aspartame", a sweet-tasting peptide, and tryptophan as an ingredient of medicines and animal feeds.

The pathway for the biosynthesis of aromatic amino acid in this organism is shown in Fig. 1. The enzyme for the first step of the common pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase, in *B. lactofermentum* and *B. flavum* was investigated biochemically.¹⁾ It was synergistically inhibited by both phenylalanine and tyrosine, and repressed by tyrosine. Furthermore, DAHP synthetase in most *m*-fluorophenylalanine resistant mutants was genetically desensitized to this synergistic inhibition.²⁾ Ito *et al.* have already cloned the gene encoding DAHP synthetase of the *m*-

fluorophenylalanine resistant mutant, *B. lactofermentum* AJ11957, and analyzed the effect of the clone on aromatic amino acid production.³⁾ However, the natures of the enzymes and genes in this common pathway in *B. lactofermentum* have not been investigated in detail except for DAHP synthetase.

In *Escherichia coli*, it is known that there are three types of DAHP synthetases⁴⁾ and two types of shikimate kinases⁵⁾ (Fig. 1). The *tyrR* gene product controls the expression of DAHP synthetases encoded by *aroF* and *aroG*, and shikimate kinase II encoded by *aroL*.^{6,7)} DAHP synthetase encoded by *aroH* is under the control of the *trpR* gene.⁸⁾ Shikimate kinase I and the other enzymes in the common pathway are synthesized constitutively except for DAHP synthetases and shikimate kinase II.⁹⁾

In *Bacillus subtilis*, the synthesis of shikimate kinase is controlled by *tyrR*, and inhibited as a part of the regulatory trifunctional enzyme complex (DAHP synthetase, choris-

mate mutase and shikimate kinase) by prephenate and chorismate.¹⁰⁾

Recently, it was reported that the synthesis

FIG. 1. Pathway for Aromatic Amino Acid Biosynthesis.

In the common pathway, thick arrows indicate the enzymes regulated through repression or feedback inhibition. Dotted lines and thin lines indicate repression and feedback inhibition, respectively. Abbreviations: PEP, phosphoenolpyruvate; ERY-4P, erythrose-4-phosphate; DHQ, dehydroquinate; DHS, dehydroshikimate; SHK, shikimate; SHK-5-P, shikimate-5-phosphate; ESP, 3-enolpyruvyl shikimate 5-phosphate; CHA, chorismate; PPA, prephenate.

of shikimate kinase in *B. lactofermentum* was repressed by tyrosine and tryptophan. Thus, shikimate kinase may also play a role as the control step in the common pathway as well as DAHP synthetases in various kinds of microorganisms. Furthermore, it has been reported that in *E. coli* mutants with unregulated DAHP synthetases, dehydroquinase becomes rate-limiting for carbon flow through the common pathway.¹¹⁾

We have developed a host-vector system for *B. lactofermentum*,¹²⁾ and have been using it successfully for gene cloning and for improvement of various amino acid productivities.¹³⁻¹⁵⁾ To investigate the structures and regulation mechanisms of the *B. lactofermentum* *aro* genes and to improve the aromatic amino acid productivities, we have cloned the *aroL* gene encoding shikimate kinase of a wild type strain, *B. lactofermentum* AJ12036. Furthermore, we have found the *aroB* and *aroE* genes, encoding dehydroquinase and shikimate dehydrogenase, respectively, on the recombinant plasmids containing the *aroL* gene.

TABLE I. BACTERIAL STRAINS EMPLOYED IN THIS STUDY

Strain	Genotype	Remarks
<i>B. lactofermentum</i> AJ12036	Prototroph	
derivatives of AJ12036		
AJ12157	<i>aroL</i>	Shikimate kinase deficient
No. 32	<i>aroE</i>	Shikimate dehydrogenase deficient
<i>E. coli</i> AB3257	<i>thi-1, ilvC7, argE3, his-4, proA2, aroH367, aroG365, aroF363, xyl-5, galK2, lacY1, mtl-1, strA712, tfr-3, tsx-358, supE44</i>	Obtained from <i>E. coli</i> Genetic Stock Center, U.S.A.
AB2847	<i>aroB351, tsx-354, mal-354, λ^R, λ⁻ supE42</i>	Obtained from <i>E. coli</i> Genetic Stock Center, U.S.A.
AB2849	<i>aroC353, tsx-357, supE42, λ⁻</i>	Obtained from <i>E. coli</i> Genetic Stock Center, U.S.A.
AB2848	<i>aroD352, tsx-356, supE42, λ⁻</i>	Obtained from <i>E. coli</i> Genetic Stock Center, U.S.A.
AB2834	<i>aroE353, mal352, tsx-352, λ^R, λ⁻ supE42</i>	Obtained from <i>E. coli</i> Genetic Stock Center, U.S.A.
AB2829	<i>aroA354, λ⁻, supE42</i>	Obtained from <i>E. coli</i> Genetic Stock Center, U.S.A.
HB101	<i>F⁻, hsdS20 (r_B⁻, m_B⁻), recA13, ara-14, proA2, lacY1, galK2, λ⁻ xyl-5, mtl-1, supE44, rpsL20 (Sm^r)</i>	Reference 26)

The *B. lactofermentum* gene designations follow the *E. coli* nomenclature.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table I. A wild type strain of *B. lactofermentum*, AJ12036,¹²⁾ was used as the DNA donor for gene cloning. Aromatic amino acid auxotrophs, *B. lactofermentum* AJ12157 (*aroL*) and No. 32 (*aroE*), were isolated from AJ12036 through *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment, and used for cloning and complementation tests.

A shuttle vector between *E. coli* and *B. lactofermentum*, pAJ1844,¹²⁾ was used for cloning of the *B. lactofermentum aro* genes. Plasmids pAJ43 (Cm^r)¹²⁾ and pUC8 (Ap^r)¹⁶⁾ were used for subcloning of each *aro* gene in *B. lactofermentum* and *E. coli*, respectively.

Media. Complete medium CM2G¹⁷⁾, Medium 31¹⁸⁾ and minimal medium MBM¹⁷⁾ were used for the cultivation of *B. lactofermentum*. For regeneration of protoplasts of *B. lactofermentum*, medium RB¹²⁾ was employed and Cm was added at 3 µg/ml, if necessary.

Davis's minimal medium¹⁹⁾ and Luria-Bertani broth²⁰⁾ were used for the cultivation of *E. coli*.

DNA isolation and recombinant plasmid construction. Total DNA of *B. lactofermentum* AJ12036 was isolated as described previously.²¹⁾ Plasmid DNAs were prepared by the slightly modified EtBr-CsCl density gradient method.²²⁾ Restriction enzymes and T4-DNA ligase were purchased from Takara and used under the conditions recommended by the supplier.

Transformation. The protoplast transformation method was employed for *B. lactofermentum*.¹²⁾ Transformation of *E. coli* was carried out as described previously.²³⁾

Preparation of cell extracts. Cells of *B. lactofermentum* were cultured in 500-ml-flasks containing 20 ml of Medium 31 at 30°C for 22 hr, harvested, washed twice with 0.2% KCl and then suspended in 0.1 M Tris-HCl buffer (pH 7.4). Then, the cells were disrupted by sonication (50 W, 8 min). The crude extract, obtained as the supernatant after centrifugation at 32,000 × *g* for 20 min, was assayed immediately for enzyme activity.

For *E. coli*, cells were cultured in 500 ml-flasks containing 100 ml of Luria-Bertani broth at 37°C for 17 hr, harvested, washed twice with 0.9% NaCl and then suspended in 0.1 M Tris-HCl buffer (pH 7.4). The cells were disrupted by sonication (50 W, 3 min) and then centrifuged at 32,000 × *g* for 20 min. The resulting crude extract was used for the enzyme assay.

Assaying of shikimate kinase activity. The incubation mixture contained 1 mM shikimate, 4 mM ATP, 5 mM MgCl₂, 10 mM NaF, 50 mM Veronal buffer (pH 9.0) and crude cell extract (0.1 to 1.0 mg of protein) in a total volume of 1.0 ml. Incubation was carried out at 30°C for

30 min. Then, 0.2 ml of 1 M Tris-HCl buffer (pH 7.8) was added, and the mixture was boiled for 2 min and then chilled on ice. Kinase activity was determined by measuring the disappearance of shikimate according to the method of Gaitonde and Gordon.²⁴⁾

Assaying of shikimate dehydrogenase activity. The method described by Yaniv and Gilvarg²⁵⁾ was used. The reaction was started by adding 2 mM shikimate to 0.1 M Tris-HCl buffer (pH 8.0), 0.2 mM NADP and enzyme (0.05 to 0.4 mg of protein) in a final volume of 1 ml. After rapid mixing, the change in absorbance at 340 µm of NADPH was measured for 3 min at room temperature.

Analysis of the copy numbers of the recombinant plasmids. The copy numbers of recombinant plasmids in *B. lactofermentum* were determined by the agarose gel electrophoresis method.²²⁾

RESULTS

Cloning of the *aroL* gene encoding the shikimate kinase of *B. lactofermentum*

Chromosomal DNA of *B. lactofermentum* AJ12036 and plasmid vector pAJ1844 was digested with *Pst*I partially and completely, respectively. They were mixed and ligated with T4-DNA ligase, and then the ligated DNA was used to transform shikimate kinase deficient mutant AJ12157 (*aroL*) which requires aromatic amino acid for growth. After incubation on regeneration medium RB supplemented with 3 µg/ml of Cm at 30°C for 10 days, about 3,800 regenerated Cm^r colonies were transferred by the replica method to minimal medium MBM. Five transformants were found to grow on MBM. From these transformants, plasmid DNAs were extracted, and designated at

FIG. 2. Restriction Enzyme Cleavage Maps of Inserts on Different Recombinant Plasmids.

Black segments indicate the common 2.9 kb *Pst*I fragment in the inserts. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; P, *Pst*I.

pAJ910, pAJ927, pAJ928, pAJ1219 and pAJ1220, respectively. These plasmids were used for re-transformation of AJ12157, and it was confirmed that all of them could complement AJ12157. Restriction enzyme cleavage maps of the inserts of these plasmids are shown in Fig. 2. When these plasmids were digested with *Pst*I, all were found to contain a common 2.9 kb *Pst*I fragment. Therefore, it was considered that the *aroL* gene encoding shikimate kinase existed on this 2.9 kb *Pst*I fragment.

Shikimate kinase activities of strains harboring recombinant plasmids containing the aroL gene

The presence of recombinant plasmids containing the *aroL* gene caused elevated levels of shikimate kinase activity in *B. lactofermentum*. The transformed strains had 14~18 copies of the recombinant plasmids and exhibited approximately 10 times higher shikimate kinase activity than that of *B. lactofermentum* AJ12036 (Table II).

Identification of the aroB and aroE genes on recombinant plasmids containing the aroL gene

To determine whether other *aro* genes existed on recombinant plasmids containing the *aroL* gene or not, each plasmid was introduced into various *aro* mutants of *E. coli*. All of the recombinant plasmids were able

to complement an *E. coli* dehydroquinase synthase deficient mutant, AB2847 (*aroB351*). Furthermore, pAJ910 and pAJ1219 (pAJ1220) were also able to complement shikimate dehydrogenase deficient mutants, *E. coli* AB2834 (*aroE353*) and *B. lactofermentum* No. 32 (*aroE*). It appeared that both pAJ927 and pAJ928 harbored the *aroL* and *aroB* genes, and that on pAJ910 and pAJ1219 (pAJ1220), the *aroL*, *aroB* and *aroE* genes existed.

Shikimate dehydrogenase activities of strains harboring recombinant plasmids containing the aroE gene

Table III shows shikimate dehydrogenase activities in *E. coli*. The presence of plasmids containing the *B. lactofermentum aroE* gene in *E. coli* AB2834 (*aroE353*) caused levels of shikimate dehydrogenase activity that were about 3-fold higher than that of *E. coli* HB101 (*aroE*⁺).²⁶⁾ On the other hand, the specific activities of shikimate dehydrogenase of *B. lactofermentum* AJ12157 harboring these plasmids exhibited levels that were about 2-fold higher than that of *B. lactofermentum* AJ12036 (data not shown). The degree of elevation of the enzyme activity was lower than that predicted on the basis of the case of shikimate kinase.

Subcloning and localization of aro genes on recombinant plasmids

The results of complementation tests and restriction enzyme cleavage maps of inserts showed that the *aroL* and *aroB* genes existed on the 2.9 kb *Pst*I fragment (Figs. 2 and 3). To subclone each of the *aroL* and *aroB* genes (Fig.

TABLE II. PLASMID COPY NUMBER AND SHIKIMATE KINASE ACTIVITY

Strain	Copy number	Specific activity (nmol/min/mg protein)
<i>B. lactofermentum</i> AJ12036		3.5
<i>B. lactofermentum</i> AJ12157		0.8
AJ12157/pAJ1844	23	0.8
AJ12157/pAJ927	18	33.5
AJ12157/pAJ928	—	37.2
AJ12157/pAJ1219	14	42.9

—, not determined.

TABLE III. SHIKIMATE DEHYDROGENASE ACTIVITY

Strain	Specific activity (nmol/min/mg protein)
<i>E. coli</i>	
HB101	5.6
AB2834	0
AB2834/pAJ927	0
AB2834/pAJ1219	16.3
AB2834/pAJ910	14.4

FIG. 3. Localization of the *B. lactofermentum aro* Genes on Cloned DNA Fragments.

Restriction enzyme cleavage maps of the subcloned DNA fragments and the results of complementation tests are shown. The dotted line in the restriction map for pAJ912 indicates the deleted region. The results of complementation tests are: + (growth) and - (no growth). The most probable location of each *aro* gene on the cloned DNA fragments is shown at the bottom of the figure. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I.

3), we at first constructed pAJ912, which has a deletion of 1.0 kb *Bgl*II fragment within the 2.9 kb *Pst*I fragment on pAJ927. pAJ912 was able to complement *B. lactofermentum* AJ12157 (*aroL*), but not *E. coli* AB2847 (*aroB351*). Then, the 1.3 kb *Pst*I-*Bam*HI fragment within the 2.9 kb *Pst*I fragment was subcloned onto *E. coli* plasmid pUC8. The resulting plasmid, pARO1, was not able to complement *E. coli* AB2847 (*aroB351*). Since pARO1 could not replicate in *B. lactofermentum*, pARO1 was digested with *Bam*HI and then cloned on pAJ43, a vector for *B. lactofermentum*. Thus, the constructed plasmid, pARO2, could complement AJ12157. Another *Pst*I-*Bam*HI fragment (1.6 kb) within the 2.9 kb *Pst*I fragment was also subcloned on pUC8, but the resulting plasmid, pARO3, could not complement *E. coli* AB2847.

On the other hand, it was suggested that the *aroE* gene is located on the 4.3 kb *Pst*I fragment of pAJ1219 and pAJ910 (Figs. 2 and 3). To determine the location of the *aroE* gene, at first the 4.3 kb *Pst*I fragment of pAJ1219 was subcloned on pAJ1844. The resulting plasmid, pARO4, could complement *E. coli* AB2834 (*aroE353*) and caused the same level of shikimate dehydrogenase activity as pAJ1219 in

AB2834. Then, a 2.0 kb *Pst*I-*Bam*HI fragment and a 3.9 kb *Eco*RI-*Pst*I fragment were prepared from the 4.3 kb *Pst*I fragment, ligated to pUC8 and then used to transform *E. coli* AB2834 (*aroE353*), respectively. The resultant recombinant plasmid, pARO5, containing the former fragment was able to complement *E. coli* AB2834, but the plasmid, pARO6, containing the latter fragment could not.

According to these results, the *aroB*, *aroE* and *aroL* genes existing on the inserts of the recombinant plasmids were localized as shown at the bottom of Fig. 3, respectively. It appeared that these three *aro* genes were located close to each other.

DISCUSSION

The *aroL* gene encoding shikimate kinase of *B. lactofermentum* was cloned. All of the recombinant plasmids isolated independently contained a common 2.9 kb *Pst*I fragment, and caused elevated levels of shikimate kinase activities in *B. lactofermentum*. In complementation tests, we have found that the *aroB* and *aroE* genes encoding dehydroquinate synthase and shikimate dehydrogenase, respectively, also existed on recombinant plasmids contain-

ing the *aroL* gene. Both the *aroB* and *aroL* genes were localized on the cloned 2.9 kb *Pst*I fragment, and the *aroE* gene was localized on the cloned 4.3 kb *Pst*I fragment, and the *aroL*, *aroB* and *aroE* genes existed close to each other on the inserts, in that order. The present results show that in *B. lactofermentum*, a Gram-positive bacterium, these three *aro* genes form a cluster on its chromosome.

In *E. coli*²⁷⁾ and *S. typhimurium*,²⁸⁾ the *aro* genes of the common pathway are scattered over their chromosomes. In *B. subtilis*, it was reported that the genes for dehydroquinase synthase, 3-enolpyruvyl shikimate 5-phosphate synthetase and one form of chorismate mutase are closely linked.²⁹⁾ However, the genes for dehydroquinase synthase, shikimate kinase and shikimate dehydrogenase are scattered over its chromosome.³⁰⁾ In contrast to the cases in these bacteria, the five central enzymes (the second through sixth enzymes) of the common pathway in *Neurospora crassa*,³¹ *Euglena*³²⁾ and *Saccharomyces pombe*³³⁾ are encoded by the *arom* gene cluster, which specifies a pentafunctional enzyme on a single polypeptide chain.

Recently, Defeyter and Pittard cloned the *aroL* gene encoding shikimate kinase II of *E. coli*, and determined the nucleotide sequence of the cloned DNA fragment.^{34,35)} They identified the *aroM* gene that was cotranscribed with *aroL*, and this *aroLM* operon was subjected to repression control by *tyrR*. The *aroM* gene encodes a 26 kilodalton gene product of unknown function. In *B. lactofermentum*, the synthesis of shikimate kinase is repressed by tyrosine. Therefore, there is a possibility that these *aroL*, *aroB* and *aroE* genes may act as an operon, but we have no genetic evidence at present. Furthermore, there is another possibility that the cloned *aroL* gene encodes a bifunctional polypeptide consisting of a shikimate kinase domain (*aroL* domain) and a dehydroquinase synthase domain (*aroB* domain).

The organization of the *B. lactofermentum* *trp* operon, and the structure and function of the control regions for expression of its *trp*

genes closely resemble those of *E. coli*.^{36,37)} This is interesting in view of taxonomy, because *B. lactofermentum* is a Gram-positive bacterium whereas *E. coli* is a Gram-negative organism. Since the cloned *aro* genes of *B. lactofermentum* were expressed in *E. coli* as well as in the case of *trp* genes,³⁸⁾ the gene expression mechanisms in *B. lactofermentum* seem to resemble those in *E. coli* generally. However, the distribution and organization of these *aro* genes on the chromosome are quite different from those in *E. coli* in contrast with in the case of the *trp* operon, and resemble those in lower eukaryotes.

The presence of recombinant plasmids containing the *aroL* gene caused elevated levels of shikimate kinase in *B. lactofermentum*. These changes in enzyme levels (approximately 10-fold) seem to reflect the copy numbers of recombinant plasmids. However, in the case of shikimate dehydrogenase, the elevation of the activity (approximately 2-fold) was lower than that of shikimate kinase activity, although both the *aroE* and *aroL* genes existed on the same plasmid. There are no available data to explain this observation.

To improve the tryptophan productivity, we have been introducing these plasmids into a tryptophan producer. Our preliminary results showed that the amplification of these genes was effective for the breeding of tryptophan producer.³⁹⁾ Sato *et al.* have also successfully improved the productivities of phenylalanine and tyrosine by introducing a recombinant plasmid containing the *aroB* and *aroL* genes of *B. lactofermentum* AJ11957 into such amino acid producers.⁴⁰⁾

REFERENCES

- 1) I. Shio, S. Sugimoto and R. Miyajima, *J. Biochem.*, **75**, 987 (1974).
- 2) S. Sugimoto, M. Nakagawa, T. Tsuchida and I. Shio, *Agric. Biol. Chem.*, **37**, 2327 (1973).
- 3) K. Sato, H. Ito, K. Matsui, K. Miwa, K. Sano, T. Tanaka, S. Nakamori and H. Enei, Abstracts of Papers, the Annual Meeting of the Society of Fermentation Technology, Japan, Tokyo, October, 1985, p. 123.
- 4) C. H. Doy and K. D. Brown, *Biochim. Biophys. Acta*,

- 104, 377 (1965).
- 5) M. B. Berlyn and N. H. Giles, *J. Bacteriol.*, **99**, 222 (1969).
- 6) K. D. Brown and R. L. Somerville, *J. Bacteriol.*, **108**, 386 (1971).
- 7) B. Ely and J. Pittard, *J. Bacteriol.*, **138**, 933 (1979).
- 8) J. Pittard, J. Camakaris and B. J. Wallace, *J. Bacteriol.*, **97**, 1242 (1969).
- 9) D. E. Tribe, H. Camakaris and J. Pittard, *J. Bacteriol.*, **127**, 1085 (1976).
- 10) E. W. Nester, J. H. Lorence and D. S. Nasser, *Biochemistry*, **6**, 1553 (1967).
- 11) T. Ogino, C. Garner and D. J. Markley, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 5828 (1982).
- 12) K. Miwa, K. Matsui, M. Terabe, K. Ito, M. Ishida, H. Takagi, S. Nakamori and K. Sano, *Gene*, **39**, 281 (1985).
- 13) S. Nakamori, M. Ishida, H. Takagi, K. Ito, K. Miwa and K. Sano, *Agric. Biol. Chem.*, **51**, 87 (1987).
- 14) Y. Morinaga, H. Takagi, M. Ishida, K. Miwa, T. Sato, S. Nakamori and K. Sano, *Agric. Biol. Chem.*, **51**, 93 (1987).
- 15) K. Sano, K. Ito, K. Miwa and S. Nakamori, *Agric. Biol. Chem.*, **51**, 597 (1987).
- 16) J. Vieira and J. Messing, *Gene*, **19**, 259 (1982).
- 17) K. Sano and I. Shiio, *J. Gen. Appl. Microbiol.*, **13**, 349 (1967).
- 18) S. Sugimoto and I. Shiio, *Agric. Biol. Chem.*, **46**, 2711 (1982).
- 19) B. D. Davis and E. S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).
- 20) J. Miller, "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972, p. 433.
- 21) H. Saito and K. Miura, *Biochim. Biophys. Acta*, **72**, 619 (1963).
- 22) K. Miwa, H. Matsui, M. Terabe, S. Nakamori, K. Sano and H. Momose, *Agric. Biol. Chem.*, **48**, 2901 (1984).
- 23) M. Mandel and A. Higa, *J. Mol. Biol.*, **53**, 159 (1970).
- 24) M. H. Gaitonde and M. W. Gordon, *J. Biol. Chem.*, **230**, 1043 (1958).
- 25) H. Yaniv and C. Gilvarg, *J. Biol. Chem.*, **213**, 787 (1955).
- 26) F. Bolivar and K. Backman, *Methods Enzymol.*, **68**, 245 (1979).
- 27) J. Pittard and B. J. Wallace, *J. Bacteriol.*, **91**, 1494 (1966).
- 28) Y. Nishioka, M. Demerec and A. Eisenstark, *Genetics*, **56**, 341 (1967).
- 29) F. Gibson and J. Pittard, *Bacteriol. Reviews*, **32**, 465 (1968).
- 30) D. J. Henner and J. A. Hoch, *Microbiol. Reviews*, **44**, 57 (1980).
- 31) N. H. Giles, M. E. Case, C. W. H. Partridge and S. I. Ahmed, *Proc. Natl. Acad. Sci. U.S.A.*, **58**, 1453 (1967).
- 32) M. B. Berlyn, S. I. Ahmed and N. H. Giles, *J. Bacteriol.*, **104**, 768 (1970).
- 33) A. Strauss, *Mol. Gen. Genet.*, **172**, 233 (1979).
- 34) R. C. Defeyter and J. Pittard, *J. Bacteriol.*, **165**, 226 (1986).
- 35) R. C. Defeyter, B. E. Davidson and J. Pittard, *J. Bacteriol.*, **165**, 233 (1986).
- 36) K. Matsui, K. Sano and E. Ohtsubo, *Nucl. Acids Res.*, **14**, 10113 (1986).
- 37) K. Sano and K. Matsui, *Gene*, **53**, 191 (1987).
- 38) K. Matsui, K. Miwa and K. Sano, *Agric. Biol. Chem.*, **51**, 823 (1987).
- 39) K. Matsui, K. Miwa and K. Sano, Abstracts of Papers, the Annual Meeting of the Society of Fermentation Technology, Japan, Tokyo, October, 1985, p. 34.
- 40) K. Sato, H. Ito, K. Matsui, K. Sano and H. Enei, Abstracts of Papers, the Annual Meeting of the Agricultural Chemical Society of Japan, Kyoto, April, 1986, p. 299.