Short Communication

CLONING OF A GENE RESPONSIBLE FOR THE SPECIFIC PRODUCTION OF AN ANTIFUNGAL ANTIBIOTIC ITURIN WITH n -C₁₆- β -AMINO ACID RESIDUE

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(Received July 21, 1992)

Bacillus subtilis NB22 was isolated from composts in our laboratory, and suppressed the growth of not only phytopathogenic fungi, but also phytopathogenic bacteria both in vitro and in vivo (11–13). This suppressive effect was attributed to an antifungal antibiotic, iturin, produced extracellularly, and this bacterium is expected to be a good candidate for a biological control agent. The structure of iturin is as follows:

where **R** is an aliphatic chain of the β -amino acid (4, 10). It contains 7 residues of D- and L- α -amino acids and a collection of β -amino acids which comprise n -C₁₄- β -amino acid, *anteiso*-C₁₅- β -amino acid, *iso*-C₁₅- β -amino acid, n -C₁₆- β -amino acid and *iso*-C₁₆- β -amino acid. Each component with n -C₁₄- β -amino acid to *iso*-C₁₆- β -amino acid corresponds to peak 1 to 5 in this order in HPLC as shown in Fig. 1A (11).

The application of genetic engineering to this antibiotic-producing strain leads to enhanced productivity of the antibiotics and also to biosynthesis of novel antibiotics by modification of the structure of the iturin. However, in comparison with biochemical investigations of peptide-antibiotics production, there have been very few studies on the molecular genetics of antibiotic production in *B. subtilis* (5, 9). To elucidate the molecular mechanisms of the production of iturin, we have

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Fig. 1. Identification of an iturin-component peak 4 by HPLC.

The precipitate at pH 2 of 100 ml of the culture supernatant of *B. subtilis* IP4 or IN18 was extracted with 10 ml of the methanol, and 20 μ l of it was analyzed in a reverse-phase column Inertsil ODS-2 (GL Sciences Inc., Tokyo, 4.6 ϕ $\times$ 250 mm) using acetonitrile–ammonium acetate (10 mM) 3:4 (v/v) as the mobile phase. The elution pattern was monitored at 280 nm and the components of iturin were separated as peaks 1 to 5 according to the elution time order. (a) 15 μ g of iturin standard sample containing 0.7 μ g of peak 4, which was purified from *B. subtilis* NB22; (b) *B. subtilis* IN18 (negative mutant); (c) IP4 (*B. subtilis* IN18 containing pIT4).

developed a highly effective transformation system for iturin producers (7,8). In this report, we describe the discovery of a gene that is involved in iturin production.

B. subtilis NB22 was grown in L medium (pH 7.2) containing 10 g of Polypepton, 5 g of yeast extract, and 5 g of NaCl per liter, and was mutagenized to obtain iturin-non-producing mutants. The cells of logarithmic growth phase were treated with 100 μ g/ml of MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) in TM buffer (50 mM Tris adjusted to pH 6.5 with maleic acid) for 30 min at 37°C. Iturin-non-producers were selected on agar plates containing a phytopathogenic fungus as follows. A phytopathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* race J1 SUF119 (12), was grown on a potato-dextrose agar (PDA) medium (potato-dextrose agar 39 g, distilled water 1 l, pH 5.6) at 30°C for 5 days and suspended in sterile distilled water. Plates were made by putting a portion of this suspension into melted L agar medium. After spotting independent colonies on this plate, iturin-non-producers were selected by the disappearance of inhibitory zones made on the lawn of the fungus. Several iturin-non-producers were obtained among 1,500 colonies tested. One stable iturin-non-producer designated IN18 was chosen and was confirmed to be a non-producer of iturin by HPLC as shown in Fig. 1B, because the sensitivity of the iturin-detection by HPLC was better than that of bioassay. This was used as a host strain for shotgun cloning of biosynthetic gene(s) of iturin.

Plasmid pTB522 (3), was chosen as the cloning vector because of its capacity

for large inserts and its characteristic of low copy number. A low copy number should avoid the possible adverse effect exerted by overexpression of the production genes in the host. Plasmid DNA extracted by the alkali lysis method (2) and chromosomal DNA from the lysate using Sarkosyl were further purified by CsCl-ethidium bromide density gradient ultracentrifugation (14). Total DNA from *B. subtilis* NB22 was partially digested with *Hind*III, and ligated to pTB522 DNA linearized with *Hind*III. The ligated DNA was used to transform *B. subtilis* IN18. Transformation of *B. subtilis* IN18 was carried out by electroporation using a Cell Porator linked to a booster (BRL Life Technologies, Inc., Gaithersburg, MD, U. S. A.) with a 0.15 cm cuvette, as described previously (8). Transformants were incubated at 30°C overnight on L agar medium with 20 µg/ml of tetracycline.

All the tetracycline-resistant transformants were replica-plated onto the L agar plate containing *F. oxysporum*, and incubated for 2 to 5 days at 30°C by inspecting inhibition zones formed against *F. oxysporum*. Out of about 12,000 transformants tested, one positive clone (IP4) exhibiting an antibiotic activity against *F. oxysporum* was isolated. When this clone was cultured in liquid No. 3 medium (10 g of Polypepton, 10 g of glucose, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 l of distilled water [pH 7.0]), only a peak 4 of iturin, which was not detected in the culture broth of the host strain IN18, was produced as shown in Fig. 1C. Although the possible emerging of other antibiotic(s) could not be denied, at least the clear recovery of the production of the peak 4 by this cloned fragment was observed. Molecular weight of this compound was determined by FAB-MS. The main molecular peak was detected at the value of 1,093, and the molecular weight of peak 4 purified from the culture broth of *B. subtilis* NB22 in the control experiment also showed the same value 1,093. If the main molecular peak was considered to be $(\text{M} + \text{Na})^+$ formed by the attachment with Na^+ , the value was the same as that determined previously (11). Actually, sodium was detected in the sample by the yellow color in flame reaction. All these results described above indicated that the strain *B. subtilis* IP4 specifically produced an iturin-component peak 4 which has the *n*- C_{16} - β -amino acid residue.

Iturin-components peaks 1 to 5 were detected from iturin producers previously isolated (11, 12), and the production ratio of each component was almost constant if the medium or cultivation conditions were controlled. Although the true reason of the specific production of peak 4 was not clear at the moment, the following speculations were possible: (i) The negative mutant IN18 had more than one defective gene, and the cloned gene could only partially complement them, and thus this made it possible to produce only the peak 4. (ii) The IN18 was defective in the ability of transferring the side chains (peaks 1–5) to the amino acids part, and the cloned gene encoded the specific carrier protein of the side chain for peak 4.

Iturin-producer IP4 harbored a 14.9 kbp plasmid (pIT4) which consisted of pTB522 and 4.4 kbp insert (Fig. 2). When this plasmid was cured from the strain IP4, no peaks of iturin were detected. Subcloning of the 4.4 kbp insert was performed. Removal of the either fragments generated from *Hind*III resulted in

Fig. 2. Restriction map of the cloned fragment.

The open parts indicate the vector plasmid pTB522 and the closed box indicates the cloned fragment. Abbreviations for restriction enzymes: H; *Hind*III, P; *Pst*I.

non-production of iturin (data not shown). Purified pIT4 was introduced into *B. subtilis* MI113 by competent cell method (1), which is a derivative of Marburg 168, an iturin-non-producer, but no iturin production was detected in this host strain. This may suggest that other gene(s) were required to produce iturin in this host strain.

Thus, we have cloned and expressed the gene which partially complements the biosynthesis of iturin. This approach of genetic complementation of the non-producing mutants should be applied to several other mutants, provided that the gene(s) of interest are spanned rather longer area or dispersed.

Although the biosynthetic pathway for iturin production is not studied at all at the moment, it is well postulated that it is synthesized through nonribosomal mechanism like most of the polypeptides antibiotics such as gramicidin S, tyrocidine, and bacitracin (5,9), because it contains residues of D- α -amino acids in its structure. In our preliminary experiments on the biosynthetic enzymes of iturin, ATP-inorganic orthophosphate (P_i) exchange reaction was suggested (unpublished observation), which was found in the biosyntheses of mycobacillin, and surfactin (6,15).

Further shotgun cloning for other biosynthetic gene(s) of iturin and analysis of the cloned fragment are now in progress based on the result of this paper.

We would like to thank Dr. T. Imanaka of Osaka University for the generous gift of plasmid pTB522, and Dr. M. Kanda of Hyogo College of Medicine for the help of assaying the enzyme activities.

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