

Cloning, Sequencing, and Characterization of the Iturin A Operon

KENJI TSUGE, TAKANORI AKIYAMA, AND MAKOTO SHODA*

Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku,
Yokohama 226-8503, Japan

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***Bacillus subtilis* RB14 is a producer of the antifungal lipopeptide iturin A. Using a transposon, we identified and cloned the iturin A synthetase operon of RB14, and the sequence of this operon was also determined. The iturin A operon spans a region that is more than 38 kb long and is composed of four open reading frames, *ituD*, *ituA*, *ituB*, and *ituC*. The *ituD* gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production. The second gene, *ituA*, encodes a 449-kDa protein that has three functional modules homologous to fatty acid synthetase, amino acid transferase, and peptide synthetase. The third gene, *ituB*, and the fourth gene, *ituC*, encode 609- and 297-kDa peptide synthetases that harbor four and two amino acid modules, respectively. Mycosubtilin, which is produced by *B. subtilis* ATCC 6633, has almost the same structure as iturin A, but the amino acids at positions 6 and 7 in the mycosubtilin sequence are D-Ser→L-Asn, while in iturin A these amino acids are inverted (i.e., D-Asn→L-Ser). Comparison of the amino acid sequences encoded by the iturin A operon and the mycosubtilin operon revealed that *ituD*, *ituA*, and *ituB* have high levels of homology to the counterpart genes *fenF* (79%), *mycA* (79%), and *mycB* (79%), respectively. Although the overall level of homology of the amino acid sequences encoded by *ituC* and *mycC*, the counterpart of *ituC*, is relatively low (64%), which indicates that there is a difference in the amino acid sequences of the two lipopeptides, the levels of homology between the putative serine adenylation domains and between the asparagine adenylation domains in the two synthetases are high (79 and 80%, respectively), implying that there is an intragenic domain change in the synthetases. The fact that the flanking sequence of the iturin A synthetase coding region was highly homologous to the flanking sequence that of *xynD* of *B. subtilis* 168 and the fact that the promoter of the iturin A operon which we identified was also conserved in an upstream sequence of *xynD* imply that horizontal transfer of this operon occurred. When the promoter was replaced by the *repU* promoter of the plasmid pUB110 replication protein, production of iturin A increased threefold.**

Many *Bacillus subtilis* strains produce a small peptide(s) with a long fatty moiety, the so-called lipopeptide antibiotics. The peptide portions of these compounds contain α -amino acids with a D configuration and are produced nonribosomally with templates of the multifunctional peptide synthetases. As in the synthesis of the peptide antibiotic gramicidin S, the peptide chain grows in a defined sequence by moving on the template of the multifunctional peptide synthetase (18, 35, 44). On the basis of the structural relationships, the lipopeptides that have been identified in *B. subtilis* are generally classified into three groups: the surfactin group (27), the plipastatin-fengycin group (16, 41, 42), and the iturin group (17). The members of the surfactin and plipastatin-fengycin groups are composed of one β -hydroxy fatty acid and 7 and 10 α -amino acids, respectively, while the members of the iturin group consist of one β -amino fatty acid and 7 α -amino acids. The presence of the β -amino fatty acid is the most striking characteristic of the iturin A group and distinguishes this group from the other two groups. The operons that encode surfactin (3), plipastatin-fengycin (16, 37, 38, 40), and mycosubtilin (4), which is a member of the iturin A group, have been sequenced and characterized. In particular, a study of the mycosubtilin operon of *B. subtilis* ATCC 6633 (4) showed that *MycA*, a novel template enzyme which has functional domain homology to β -ketoacyl syn-

thetase and amino transferase and amino adenylation, was present, which implied that *MycA* is responsible for incorporation of the β -amino fatty acid.

Recently, biological control agents for plant diseases have received considerable attention as alternatives to chemical pesticides (32). *B. subtilis* RB14, which has a suppressive effect against several phytopathogens, is expected to be used as a biocontrol agent (1, 5). We previously demonstrated that the biocontrol activity of RB14 can be attributed mainly to production of iturin A (Fig. 1) (10, 26). Iturin A, as well as mycosubtilin, is a member of the iturin group. The amino acid compositions of iturin A and mycosubtilin are almost identical, except that the sixth and seventh amino acids are inverted, as shown in Fig. 1. In our previous study, we cloned a gene, *lpa-14*, that encodes the 4'-phosphopanthetheinyl transferase required for maturation of the template enzyme of iturin A (6, 8, 15). For further investigation of the details of the synthesis steps and because of the interesting evolutionary relationship between iturin A and mycosubtilin, cloning and sequencing of the complete iturin A synthetase operon are essential.

In this study, we examined the features of the iturin A synthetase operon based on the nucleotide sequence and gene disruption. By comparing the iturin A operon with the mycosubtilin operon, we found that the difference between the two operons may be a result of intragenic swapping of amino acid adenylation domains. We also obtained genetic evidence of probable horizontal transfer of the iturin A operon. In addition, a promoter replacement experiment whose goal was construction of a iturin A hyperproducer is also described below.

* Corresponding author. Mailing address: Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan. Phone: 81-45-924-5274. Fax: 81-45-924-5276. E-mail: mshoda@res.titech.ac.jp.

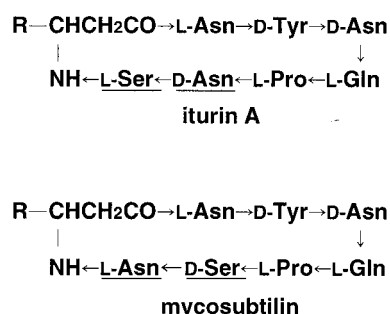


FIG. 1. Structures of iturin A and mycosubtilin. R indicates an alkyl moiety (generally C₁₄ to C₁₇). The arrows represent peptide bonds in the -CO-NH- direction. The differences between the two lipopeptides are indicated by underlining.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium was used for cultivation of *Escherichia coli* and *B. subtilis* (30). When necessary, antibiotics were added at the following concentrations: ampicillin, 50 µg/ml; chloramphenicol, 5 µg/ml; erythromycin, 10 µg/ml; tetracycline, 20 µg/ml; and neomycin, 20 µg/ml.

Iturin A production in vitro was indicated by the formation of a clear inhibitory zone on LB agar (LB medium with 1.5% agar) containing a spore suspension of a phytopathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* race J1 SUF119, as described previously (5). Number 3 medium was used for lipopeptide production in liquid cultures and contained (per liter) 10 g of Polypepton (Nihon Pharmaceutical Co., Tokyo, Japan), 10 g of glucose, 1 g of KH₂PO₄, and 0.5 g of MgSO₄ · 7H₂O (pH 6.8). Number 3S medium contained 10 g of Polypepton S (Nihon Pharmaceutical Co.) per liter instead of Polypepton because iturin A productivity was enhanced by Polypepton S.

Transformation and DNA manipulation. *B. subtilis* RB14 was transformed by electroporation as described previously (19, 39). *B. subtilis* MI113 was transformed by a competent cell method as described previously (39). Chromosomal

DNA of *B. subtilis* strains were prepared by using a method developed by Itaya and Tanaka (12). Routine DNA manipulation and *E. coli* transformation were performed as described previously (39). Plaque and Southern hybridizations were performed by a digoxigenin enzyme-linked immunosorbent assay using a DNA labeling and detection kit (Roche) as recommended in the instruction manual.

Transposon technique. Transposon-harboring plasmid pHV1249 (25) was introduced into RB14 by electroporation, and random mutagenesis of mini-Tn10 was performed by a previously described method (25). Cloning of the mini-Tn10-disrupted gene was carried out as follows. To construct a *B. subtilis* MI113 plasmid library of the RB14 chromosome, chromosomal DNA of the transposon-containing mutant was digested with *Hind*III, ligated at the *Hind*III site of plasmid pTB522 which could be replicated in *B. subtilis* (9), and then transformed into *B. subtilis* MI113 competent cells. The plasmid of the chloramphenicol-resistant transformant resulting from cloning of a chromosomal fragment containing mini-Tn10 was obtained from the library and designated pTB1006. The *Hind*III insert of pTB1006 was transplanted into the *Hind*III site of pBR322 by transformation of *E. coli* JM109, resulting in pBRHd8k (Fig. 2).

Phage library construction and screening. The RB14 chromosome was digested partially with *Eco*RI and then was separated by electrophoresis to obtain homogeneous fragments that were 10 to 20 kb long. The fragments obtained were used for lambda DASH II library construction with a Lambda DASH II/*Eco*RI vector kit (Stratagene) as recommended in the instruction manual. First, screening by plaque hybridization in which the 1.9-kb *Nde*I-*Pst*I fragment of pBRHd8k was used as a probe identified a positive phage with a 19.2-kb insert, designated λ12 (Fig. 2). By using the internal 1.7-kb *Eco*RI-*Hind*III fragment of λ12 as a probe, λ66, harboring a 16.0-kb insert, was obtained (Fig. 2). The end of the 1.9-kb *Eco*RI fragment of λ66 was employed in the next screening, and λ69 with a 12.4-kb insert was obtained (Fig. 2).

Disruption of *ituD* by the plasmid pop in-pop out method. A 2-kb *Sau*3AI-*Pst*I fragment from pBRHd8k, which harbored the entire *ituD* gene, was inserted at the *Bam*HI-*Pst*I site of pUC19, generating pSC24Pst. The *Sal*I-*Nsi*I fragment of pSC24Pst was removed, and the *Sal*I-*Pst*I fragment of the neomycin resistance gene cassette (*neo*) from pBEST502 was inserted (11), resulting in pSC24PstNm. The *Hind*III-*Kpn*I fragment of pSC24PstNm, which harbored the disrupted *ituD* gene, was inserted at the *Pst*I site of pE194 by blunt-end ligation. The ligated mixture was transformed into MI113. We selected a neomycin-resistant colony and thus obtained pE24PstNmα. The *ituD* coding region in RB14 was disrupted

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic ^a	Reference
<i>E. coli</i> JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 Δ(lac-proAB) /F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	30
<i>B. subtilis</i> strains		
RB14	Wild type, IT ⁺ SF ⁺ PL ⁺	5
1006	RB14 <i>ituA</i> ::Tn10, IT ⁻ SF ⁺ PL ⁺	This study
RΔIA1	RB14 <i>ituD</i> :: <i>neo</i> , IT ⁻ SF ⁺ PL ⁺	This study
R-PM1	RB14 P _{repU-neo-ituD} , IT ⁺ SF ⁺ PL ⁺	This study
MI113	Marburg 168 derivative, <i>arg-15 trpC2 hsmM hsrM</i>	40
<i>E. coli</i> plasmids		
pUC19	Cloning vector, Ap ^r	30
pBEST502	P _{repU-neo} cassette, Ap ^r Nm ^r	11
pSC24Pst	pUC19 carrying the 2-kb <i>Sau</i> 3AI- <i>Pst</i> I fragment containing <i>ituD</i> , Ap ^r	This study
pSC24PstNm	Nm ^r gene cassette inserted into the internal <i>Sal</i> I- <i>Nsi</i> I region of <i>ituD</i> in pSC24Pst, Ap ^r Nm ^r	This study
pSC53EcoT	pUC19 carrying the 1.2-kb <i>Tth</i> HB8I- <i>Eco</i> T14I fragment containing P _{ituD} , Ap ^r	This study
pKODP4P5	pUC19 carrying the 0.8-kb PCR product containing 5'-terminal region of <i>ituD</i> as well as the putative ribosome binding site, Ap ^r	This study
pP4P5Nm	P _{repU-neo} inserted into the <i>Xba</i> I site located upstream of the putative ribosome binding site of <i>ituD</i> in pKODP4P5, Ap ^r Nm ^r	This study
pUCIPNm	pP4P5Nm carrying the 0.8-kb <i>Nde</i> I- <i>Tth</i> HB8I fragment containing 3'-terminal region of <i>yxjF</i> , Ap ^r Nm ^r	This study
<i>B. subtilis</i> plasmids		
pHV1249	Mini-Tn10 Ap ^r Em ^r Cm ^r	25
pTB522	Cloning vector, Tc ^r	9
pE194	Temperature sensitive for replication, Em ^r	40
pE24PstNmα	Disruption plasmid, pE194 carrying the 2.0-kb insert from pSC24PstNm, <i>ituD</i> :: <i>neo</i> gene cassette, Em ^r Nm ^r	This study
pEIPNm2	pE194 carrying the 3.0-kb insert from pUCIPNm, P _{ituD-neo-ituD} , Em ^r Nm ^r	This study

^a IT, iturin A production; SF, surfactin production; PL, plipastatin-like antifungal agent production; Ap^r, ampicillin resistant; Em^r, erythromycin resistant; Nm^r, neomycin resistant; Tc^r, tetracycline resistant.

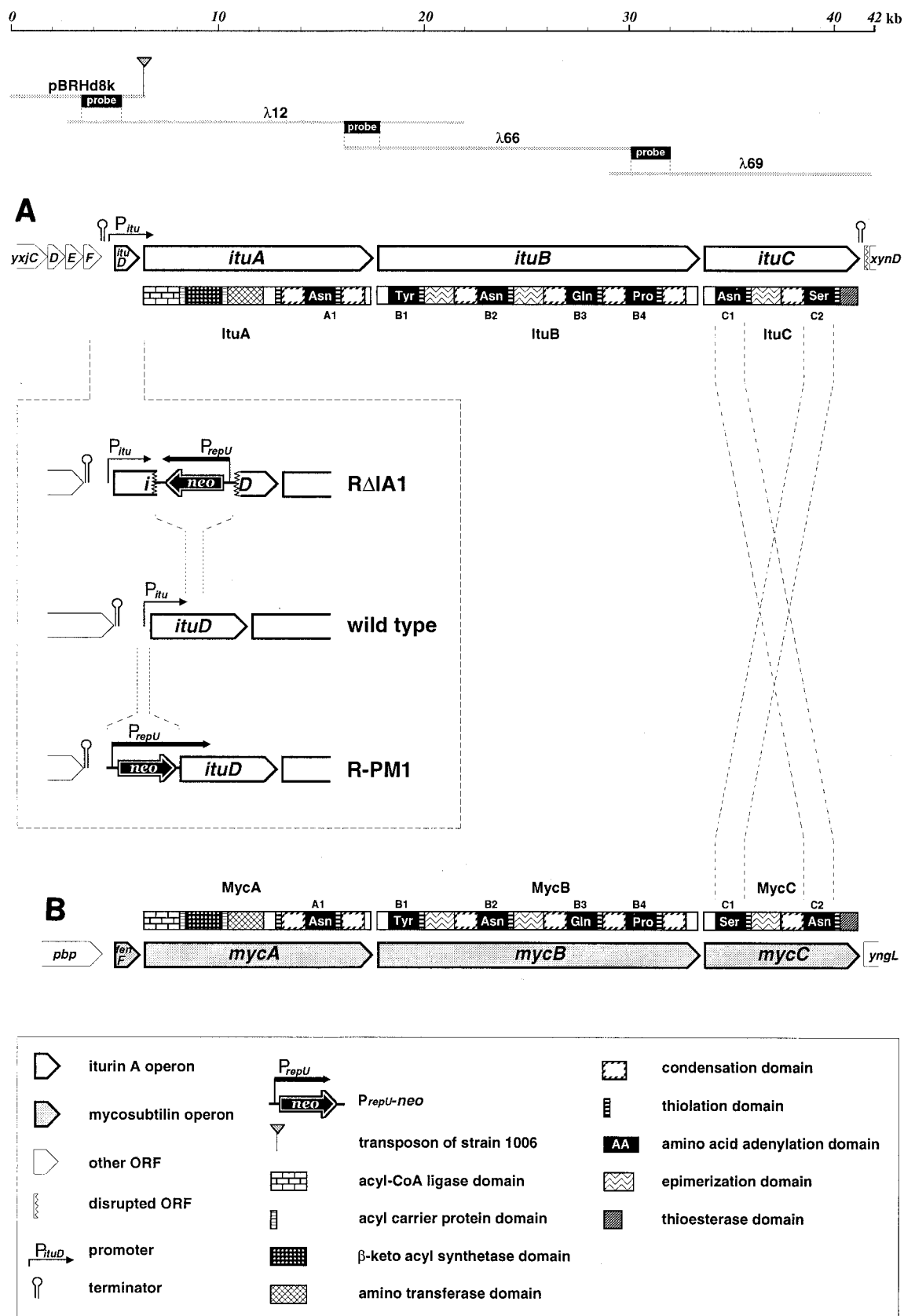


FIG. 2. ORF organization of iturin A (A) and mycosubtilin (B) operons. The positions of the plasmid and phages sequenced are shown above the iturin A operon. The sequences of derivative strains R Δ IA1 and R-PM1 associated with *ituD* are shown in a box. The intersecting dotted lines indicate the difference in the amino acid adenylation domain arrangement between the two operons. The mycosubtilin operon was drawn by referring to reference 4.

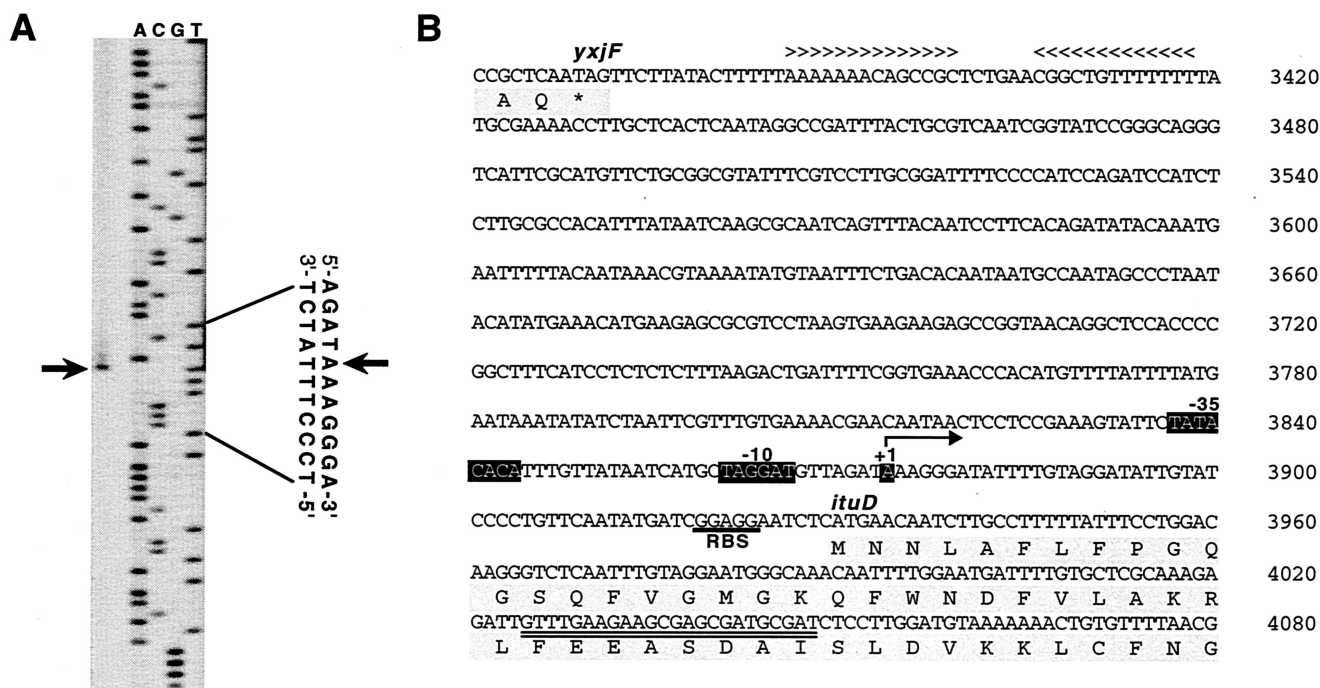


FIG. 3. Determination of the transcription initiation site of *ituD* by primer extension analysis. (A) The position of the band corresponding to the *ituD*-specific primer extension product is indicated by arrows. (B) Nucleotide sequence of the *ituD* promoter region (P_{ituD}). The positions of the *ituD* transcription initiation site (highlighted, +1), putative -10 and -35 regions (highlighted), and putative ribosome binding site (RBS) (underlined) are indicated. Putative protein sequences of YxjF and ItuD are shown below the DNA sequence (shaded). The sequence complementary to the oligonucleotide used for primer extension analysis (ITU-PREX) is double underlined. The putative ρ -independent terminator downstream of *yxjF* is indicated by > and < above the sequence.

by using the thermosensitive replication origin of pE194, as described previously (40). First, pE24PstNm α was transformed into RB14 by electroporation. Strain RB14(pE24PstNm α) was plated onto LB agar containing neomycin and erythromycin and then incubated at 48°C. The resulting strain, RB14::pE24PstNm α , was cultivated in LB medium without selective pressure at 30°C for 10 generations. The culture was diluted and plated onto LB agar to obtain single colonies. The neomycin resistance and erythromycin resistance of the resulting colonies were assayed. Finally, the disrupted mutant, Δ IAl, was isolated by screening for neomycin-resistant and erythromycin-sensitive colonies.

Primer extension analysis. Total mRNA was prepared from a stationary-phase culture of RB14 cultivated in Number 3 medium for 17 h. Four milliliters of the culture was centrifuged and washed with STE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.1 M NaCl). The pellet was then resuspended in 50 μ l of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and 50 μ l of TE buffer containing lysozyme (20 mg/ml) was added. The suspension was incubated at room temperature for 10 min. Further purification was performed with an RNeasy mini kit (Qiagen). For primer extension analysis, IRD41-labeled primer ITU-PREX (5'-ATCGCATCGCTCGCTTCTCAAAC-3') (Fig. 3), which was complementary to the sequence from nucleotide 96 to nucleotide 119 downstream of the putative start codon of *ituD*, was purchased from NisSHINBO Co. (Tokyo, Japan). Total RNA was ethanol precipitated and dissolved in 20 μ l of hybridization buffer [80 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.4), 2 mM EDTA, 800 mM NaCl, 50% formamide]. The solution was supplemented with 1.8 μ l of primer ITU-PREX (1 pmol/ μ l), denatured at 80°C for 15 min, and then cooled to 30°C with gentle shaking. After ethanol precipitation, the pellet was dissolved in extension buffer (4 μ l of Moloney murine leukemia virus reverse transcriptase [Toyobo Inc., Osaka, Japan], 8 μ l of 5 \times buffer for Moloney murine leukemia virus reverse transcriptase, 16 μ l of a solution containing each deoxynucleoside triphosphate at a concentration of 2.5 mM, 10 μ l of H₂O, 2 μ l of RNase inhibitor) and then incubated at 42°C for 1 h. The resulting cDNA was subjected to Li-cor dNA automated DNA sequencing.

Promoter exchange. A 0.8-kb fragment of the 5' region of *ituD*, which contained up to the ribosome binding site but not the promoter, was obtained by PCR. PCR amplification with primers ITUP4-F (5'-CCCTGTCTCATGATGA

TCCGAGGAATCTC-3'; underlining indicates an *Xba*I site, and italics indicates substituted bases) and ITUP5-R (5'-TGCATCGATTCTGTCCATCTAACCGGCATC-3'; underlining indicates a *Cla*I site) was performed with the RB14 chromosome by using KOD DNA polymerase (Toyobo Inc.). The fragment obtained was double digested with *Xba*I and *Cla*I and then inserted between the *Xba*I and *Cla*I sites of pUC19. The resulting plasmid was confirmed to have the correct sequence and was designated pKODP4P5. P_{repU} accompanied by the neomycin resistance gene cassette (P_{repU} -*neo*) was excised from pBEST502 (11) by digestion with *Xba*I and then inserted into the *Xba*I site of pKODP4P5. A plasmid with P_{repU} -*neo*, whose direction of transcription is the same as that of *ituD*, was selected and designated pP4P5Nm. The 0.8-kb *Nde*I-*Tth*HB8I fragment, which was upstream of the *ituD* transcription start site prepared from plasmid subclone pS53EcoT of the phage library, was blunt ended and inserted into the *Sma*I site of pP4P5Nm, generating pUCIPNm. The *Hind*III-*Kpn*I fragment of pUCIPNm, which was P_{repU} -*neo* accompanied by the P_{itu} flanking region, was inserted into the *Pst*I site of pE194 by blunting and ligation and then transformed into MI113, which resulted in plasmid pEIPNm2. Replacement of P_{itu} of RB14 by P_{repU} -*neo* by the plasmid pop in-pop out method using pEIPNm2 was performed by the method described above for *ituD* disruption. Promoter replacement was confirmed by PCR (data not shown).

Quantitative analysis of iturin A and surfactin. A culture of *B. subtilis* in 40 ml of Number 3 medium was acidified to pH 2.0 with 12 N HCl. Then the precipitate was collected by centrifugation and extracted with methanol. Iturin A and surfactin in the extracted solution were quantified by reversed-phase high-performance liquid chromatography (HPLC), as described previously (1, 40).

Nucleotide sequence analysis. Double-stranded DNA cloned in pUC19 was sequenced with a Li-cor dNA 4000L DNA sequencer by using an IRD41 dye-labeled primer (Nisshinbo Co., Tokyo, Japan) and a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech Co.). A partial sequence of the insert of the screened lambda DASH II phage was determined by Nippon Seifun Co., Atsugi, Japan. Motif retrieval for proteins was performed by using the PROSITE package. Harplot analysis was performed with the Genetyx package (Software Development Co., Tokyo, Japan). Multiple-alignment analysis and phylogenetic analysis were performed by using Clustal W.

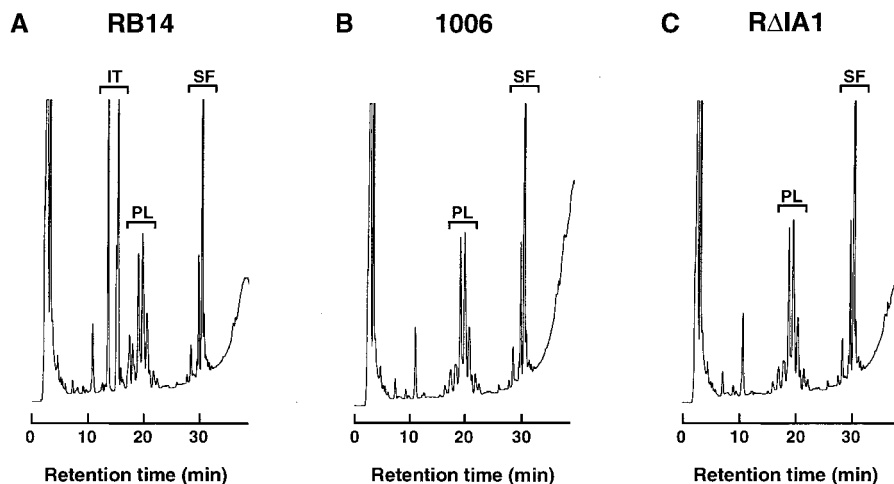


FIG. 4. Qualitative HPLC analysis of the lipopeptides produced by RB14 (A), 1006 (B), and $R\Delta IA1$ (C). Peaks corresponding to iturin A (IT) and surfactin (SF) are indicated. Plipastatin-like peaks (not identified) (PL) are also indicated.

Nucleotide sequence accession number. The nucleotide sequence of the iturin A operon of RB14 has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB050629.

RESULTS

Cloning, sequencing, and analysis of iturin A operon. To identify the genes responsible for iturin A production, we performed transposon mutagenesis with mini-Tn10. About 5,000 transposon-containing colonies were replicated on an LB agar plate containing *F. oxysporum* to assay for iturin A production deficiency. Fifteen colonies exhibited no antifungal activity on the plate, and none of these colonies had the ability to produce iturin A, as determined by HPLC. Ten of the colonies were selected at random, and chromosomal DNA were prepared, digested by *Hind*III, and subjected to Southern hybridization. All 10 colonies contained mini-Tn10 in the same 8-kb *Hind*III fragment (data not shown). We selected one colony, designated strain 1006, for further study. No iturin A production by 1006 was observed (Fig. 4). The 8-kb *Hind*III fragment with a transposon was screened from the plasmid library of the strain 1006 chromosome by using the chloramphenicol resistance marker of mini-Tn10.

To clone a complete iturin A operon, an RB14 chromosome library was constructed by using the lambda DASH II phage, and four contiguous inserts that collectively encompassed a 42-kb region were obtained. Figure 2 shows that this region contains nine open reading frames (ORFs). All nine ORFs are oriented in the same direction. The four ORFs located upstream in the region sequenced have high levels of homology to the *B. subtilis* 168 genes *yxjC* (77% identical), *yxjD* (83%), *yxjE* (83%), and *yxjF* (77%). These genes are thought to encode 3-oxoadipate coenzyme A (CoA) transferase (*yxjD* and *yxjE*) and gluconate 5-dehydrogenase (*yxjF*) (14), but their actual functions have not been determined. There is a putative rho-independent terminator downstream of *yxjF* that appears to terminate transcription from both directions. The fifth ORF, designated *ituD*, is separated from *yxjF* by a 0.6-kb intercoding region. The 45-kDa ItuD protein has a high level of homology to FenF encoded by the mycosubtilin synthetase operon of *B.*

subtilis ATCC 6633 (79%) (4) and FenF of *B. subtilis* F29-3 (89%) (2) and lower levels of homology to malonyl-CoA transacylase of *E. coli* (44%) (43) and *B. subtilis* 168 (37%) (23), suggesting that *ituD* encodes malonyl-CoA transacylase. The transposon-containing ORF, located 19 bp downstream of *ituD*, is designated *ituA*. The deduced amino acid sequence encoded by *ituA* corresponds to a 449-kDa protein that has a high level of homology to MycA (79%), which is the first subunit of mycosubtilin synthetase. The striking feature of MycA, the fact that three functional domains homologous to β -ketoacyl synthetase, amino transferase, and amino acid adenylation are combined, is conserved in ItuA. The gene 43 bp downstream of *ituA* is called *ituB*. ItuB is a 609-kDa peptide synthetase consisting of four amino acid adenylation domains, two of which are flanked by an epimerization domain. ItuB also exhibits 79% homology to MycB. The next gene is designated *ituC*, which encodes a peptide synthetase that has two adenylation domains, one epimerization domain, and a thioesterase domain that is probably responsible for peptide cyclization. Although ItuD, ItuA, and ItuB have high levels of homology to their counterparts in mycosubtilin synthetase, ItuC exhibits only 64% homology to MycC, which may reflect a structural difference between iturin A and mycosubtilin. There is a putative rho-independent terminator downstream of *ituC*. The final ORF exhibits homology to the *xynD* gene (29) of *B. subtilis* 168, which encodes endo-1,4-xylanase. As shown in Fig. 5, there is a sequence that has a high level of homology to the *xynD* gene and is located between *ituC* and *xynD* in a direct-repeat manner (see below).

Disruption of the *ituD* gene to derive the iturin A-deficient phenotype. To confirm that *ituD* is responsible for iturin A synthesis, *ituD* was disrupted (Fig. 2). The resulting disruptant, $R\Delta IA1$, was inoculated into Number 3S medium and cultivated at 30°C for 60 h to assay for iturin A production. As shown in Fig. 4, analytical HPLC of an $R\Delta IA1$ culture extract resulted in no iturin A peak, while the production of the lipopeptide surfactin by $R\Delta IA1$ was the same as the production by the wild type, indicating that *ituD* disruption only re-

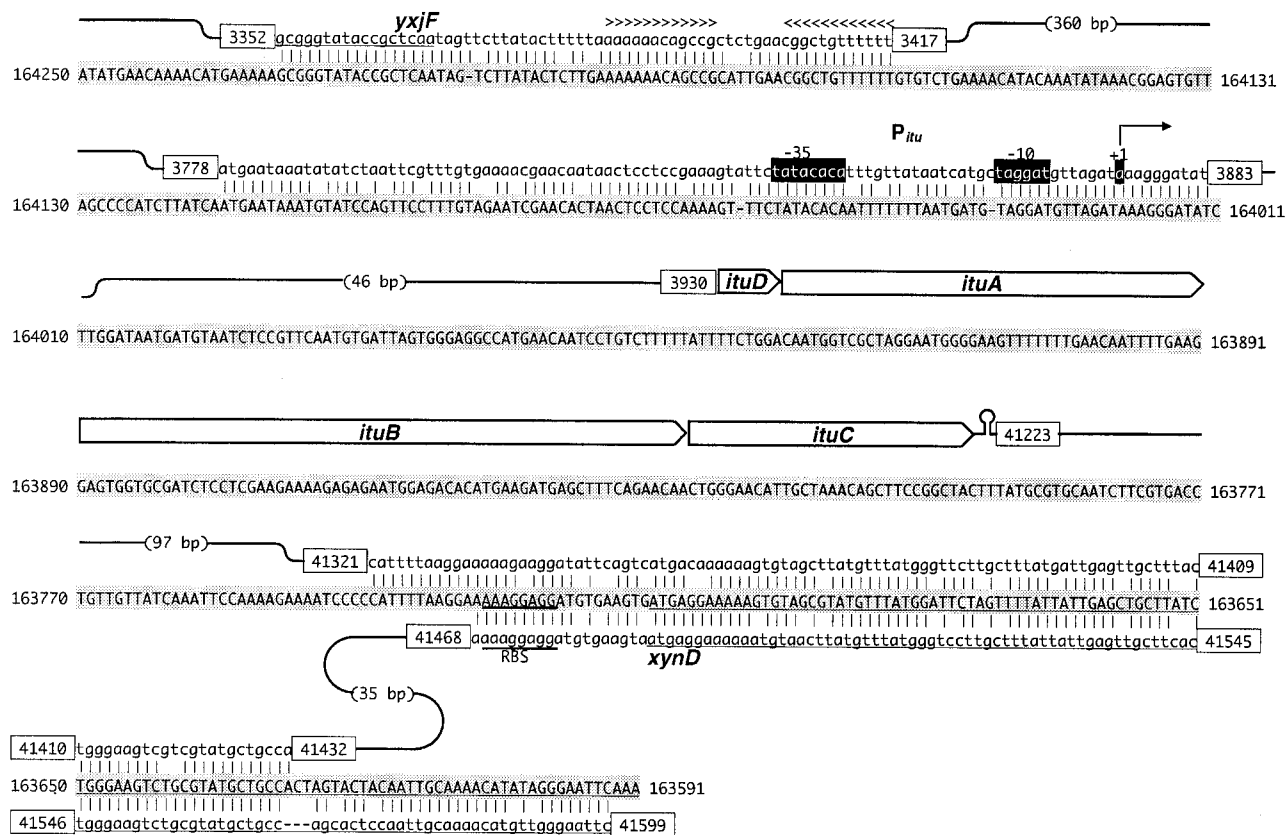


FIG. 5. Alignment of iturin A operon of RB14 and *xynD* regions of strain 168. Shaded uppercase letters indicate the sequence of the *xynD* region of strain 168 (based on the complementary sequence from positions 164, 250 to 163, 691 of the accession no. Z99113 sequence). Lowercase letters indicate the iturin A operon sequence (accession no. AB050629) that has homology to the strain 168 sequence. Sequences encoding iturin A synthetase are indicated by arrow-shaped boxes. Identical nucleotides in the two sequences are indicated by vertical lines. The numbers in the boxes are the positions in the deposited sequence. The lines indicate linkage between discrete homologous segments of the iturin A operon. Underlining indicates coding regions of *yxjF* and *xynD*. -35 and -10 are promoter sites of *ituD*, and $+1$ is a transcription start site of *ituD*. The alignment was constructed based on the results of a BLAST search. RBS, ribosome binding site.

sulted in an iturin A deficiency. Based on these results, we concluded that *ituD* is essential specifically for iturin A synthesis.

Promoter analysis of the *ituD* gene. The transcription start site of *ituD* was determined by the primer extension method. Total RNA of RB14 was extracted from a 17-h culture in Number 3 medium. The total RNA obtained was hybridized with oligoDNA by using a fluorescent probe that was designed to hybridize with nucleotides 96 to 119 of *ituD* from the translation initiation codon. cDNA was synthesized with reverse transcriptase, and the resulting cDNA was then sequenced. As shown in Fig. 3, the transcription start site of *ituD* was found to be an A residue 56 bp upstream of the first residue of the *ituD* initiation codon. Upstream of this start site, we found a TATACACA-16 bp-TAGGAT sequence that exhibited low levels of homology to the consensus -10 and -35 (TTGACA-17 bp-TATAAT) sequences of σ^A (Fig. 3). This promoter was designated P_{itu} . We also searched for other transcription start sites up to the putative rho-independent terminator of *yxjF* but did not find any other such sites.

Promoter replacement of the iturin A operon for high iturin A production. Because the mutant with disrupted *ituD* had an iturin A deficient phenotype and there is no coding region

between *ituD* and *iturC*, which implies that there is a promoter, it is possible that P_{itu} governs expression of the iturin A operon. To confirm this, we replaced P_{itu} with a constitutively expressed promoter by using the neomycin resistance gene cassette, $P_{repU-neo}$, of pBEST502 (10). $P_{repU-neo}$ is the chimera of the promoter of the replication protein gene (P_{repU}) and the neomycin resistance gene (*neo*) of plasmid pUB110 (20). Since P_{repU} of $P_{repU-neo}$ lacks autoregulation of transcription by RepU (24), constitutive expression of P_{repU} is expected. Moreover, P_{repU} is sufficiently strong that neomycin resistance can be conferred by a single copy of *neo* in the chromosome, and there is no terminator to stop transcription starting from P_{repU} . The presence of the neomycin resistance gene cassette in a transformant, therefore, indicates that P_{repU} has been introduced and implies that constitutive and enforced expression of genes located downstream of $P_{repU-neo}$ occurs in a polycistronic manner. The neomycin resistance gene cassette was ligated with a PCR-amplified fragment of the 5' portion of *ituD* at the ribosome binding site of *ituD* and then attached to the upstream fragment of P_{itu} in front of the neomycin resistance gene. The resulting three-fragment array was ligated to the thermosensitive plasmid pE194 and transformed by electroporation, and

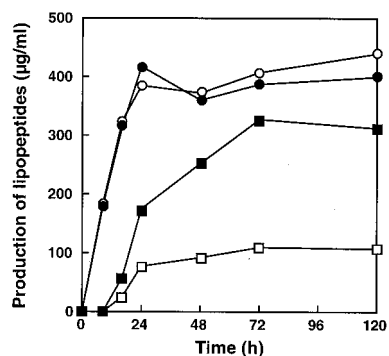


FIG. 6. Time courses for production of two lipopeptides, iturin A produced by RB14 (□) and R-PM1 (■) and surfactin produced by RB14 (○) and R-PM1 (●).

then the promoter was replaced by the pop in-pop out method, as described above for *ituA* disruption. The resulting strain, R-PM1, contained the inserted $P_{repU-neo}$ instead of 250 bp of the P_{itu} sequence (Fig. 2).

The strain generated and the wild type were inoculated into Number 3 medium and cultured at 30°C for 120 h to monitor iturin A production. The changes in the growth of strain R-PM1 and the pH of Number 3 medium over time were the same as those observed with the wild type (data not shown). However, as shown in Fig. 6, the rate of iturin A production by R-PM1 was significantly greater than that by the wild type. The concentration of iturin A for R-PM1 after 72 h of cultivation was 330 µg/ml, whereas the concentration for the wild type was 110 µg/ml, indicating that production was threefold greater in R-PM1. Since the two strains did not differ significantly in terms of surfactin production (Fig. 6), we concluded that the promoter exchange affected only iturin A production.

DISCUSSION

In this study, we identified the 42-kb region of *B. subtilis* RB14 that contains the complete iturin A synthetase operon, which is more than 38 kb long. The iturin A operon is composed of four ORFs, *ituD*, *ituA*, *ituB*, and *ituC*, in that order. The iturin A operon closely resembles the mycosubtilin operon. *ItuD*, as well as *FenF* of *B. subtilis* ATCC 6633 (4) and F29-3 (2), exhibits homology to the malonyl CoA-transacylase *FabD*, which participates in fatty acid synthesis in *E. coli* (43) and *B. subtilis* 168 (23). It is thought that in some *Streptomyces* spp. malonyl CoA-transacylase may be responsible for not only fatty acid synthesis but also type II polyketide antibiotic synthesis (28, 36). To clarify whether *ItuD* is involved in iturin A production and/or fatty acid synthesis, we disrupted *ituD*, which resulted in a specific iturin A deficiency. Since neither the growth of nor surfactin production by strain RΔIA1, a strain in which *ituD* was disrupted, was inhibited, we concluded that *ituD* is indispensable for iturin A production. This is the first evidence that a gene related to *fenF* participates in synthesis of an iturin group antibiotic. On the other hand, *ItuA* exhibits homology to β-ketoacyl synthetase, amino transferase, and peptide synthetase in one molecule and is probably responsible for synthesis of a β-amino fatty acid accompanied by *ItuD* dipeptide (β-amino fatty acid-Asn) formation, as has

been proposed for the mycosubtilin synthetase *MycA* (4). These features are consistent with the report that cerulenin, which inhibits β-ketoacyl synthetase, is more active in iturin A β-amino fatty acid synthesis than in fatty acid synthesis, which implies that β-ketoacyl synthesis occurs during iturin A production independent of fatty acid synthesis (7). Other genes, *ituB* and *ituC*, encode large peptide synthetases that build peptide chains on the precursor from *ItuA*. The level of homology between *ituC* and *mycC*, the counterpart of *ituC* in the mycosubtilin operon, is relatively low (64%) compared to the levels of homology for other groups (79%), reflecting the difference in the amino acid arrangements in *ItuC* and *MycC*. As shown in Fig. 2, *ItuC* is predicted to be responsible for the D-Asn→L-Ser portion of iturin A, while *MycC* is thought to synthesize the D-Ser→L-Asn part of mycosubtilin. Therefore, it is not unreasonable to assume that swapping between two adenylation domains occurs. To examine the possibility that intragenic swapping occurs in the adenylation domain, the sequences of *ituC* and *mycC* were compared. The results of a dot matrix analysis of the nucleotide sequences of *ituC* and the other relevant synthetase genes (*mycC*, *ituA*, and *ituB*), including *ituC* itself, are shown in Fig. 7. Clearly, two regions of *ituC* exhibit discrete homology to *mycC*; the homology junction is roughly 10 amino acids upstream of L(TS)xEL (A1 motif sequence [18]) and the C terminus of GRxDxQVKIRGxRIELG EIE (A8 motif sequence [18]). Homologies are observed between asparagine adenylation domains (*ItuC2* and *MycC1*) and between serine adenylation domains (*ItuC1* and *MycC2*), implying that domain swapping occurs. Since there are three asparagine adenylation domains (*ItuA1*, *ItuB2*, and *ItuC1*) in iturin A synthetases (Fig. 2), it is likely that the asparagine adenylation domain of *ItuC1* exhibits the highest level of homology to *ItuA1* or *Itu2*. However, as shown in Fig. 8, phylogenetic analysis of 14 nucleotide sequences encoding the adenylation domain of iturin A and mycosubtilin revealed that the sequence most similar to the sequence encoding the asparagine adenylation domain of *ItuC1* is the sequence encoding the asparagine adenylation domain of *MycC2*. All iturin A domains also show phylogenetic similarities to their counterparts in mycosubtilin domains. These results imply that iturin A and mycosubtilin have a common ancestor. Therefore, we propose a model in which *ituC* or *mycC* swapped nucleotide sequences encoding adenylation domains after a common ancestor became established.

Although the overall sequence of the iturin A operon is highly homologous to that of the mycosubtilin operon, the flanking region of the iturin A operon is significantly different from that of the mycosubtilin operon. It has been shown that the mycosubtilin operon of ATCC 6633, which is not a plipastatin producer, is between *pbp* and *yngL* (4), while the plipastatin operon (167° to 171°), instead of the mycosubtilin operon, is present in strain 168 (40). In the case of RB14, the iturin A operon lies between *yxjF* and *xynD*. Since strain RB14 is also a producer of a plipastatin-like compound (Fig. 4), it is reasonable to conclude that the iturin A operon of RB14 is in a region other than the plipastatin region. However, in the case of strain 168, *yxjF* (341°) is located on the side opposite *xynD* (166°) in the chromosome (14). This can be explained by the fact that the *SfiI* digestion pattern of the RB14 genome, as determined by pulsed-field gel electrophoresis, was signifi-

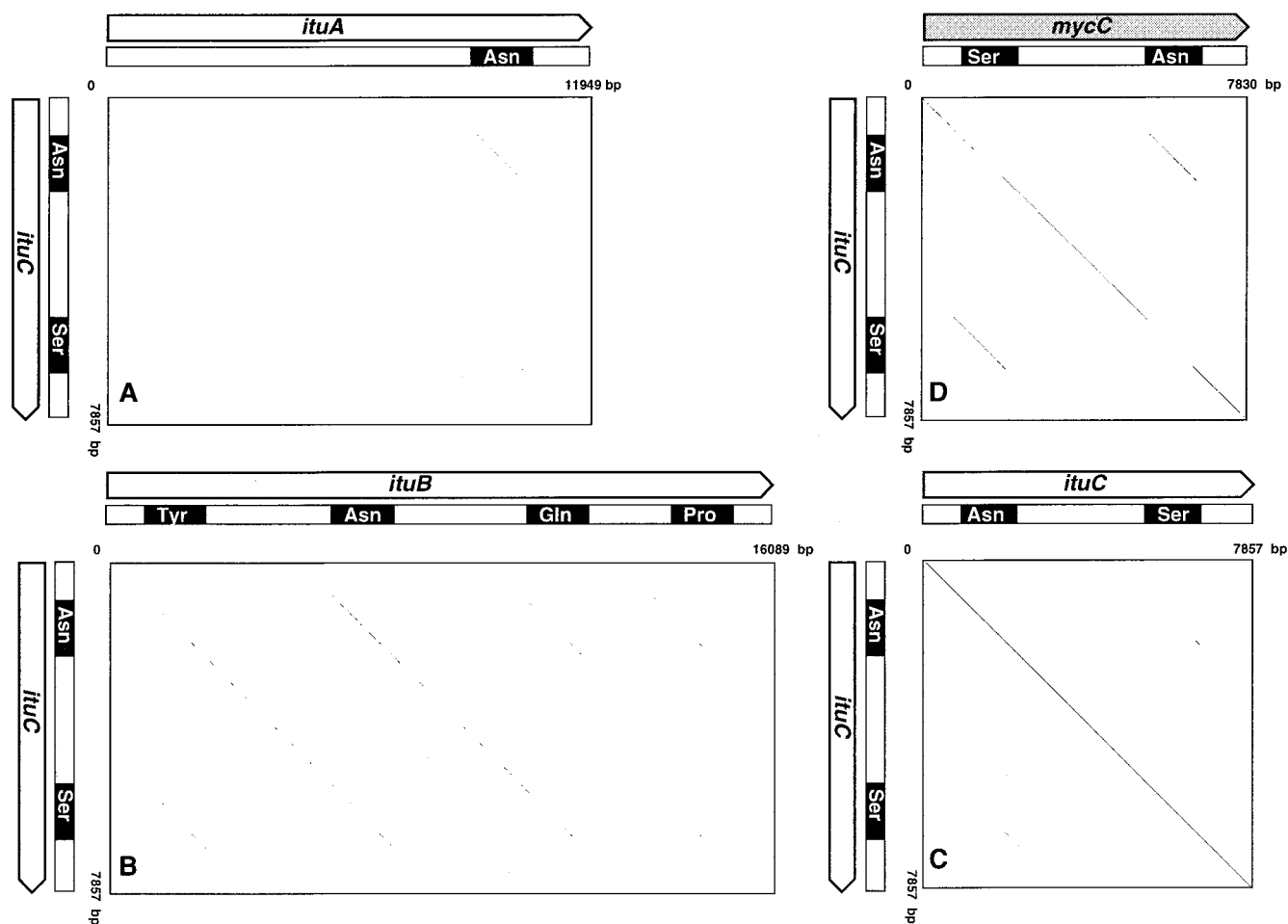


FIG. 7. Dot matrices for *ituC* and *ituA* (A), *ituB* (B), *ituC* (C), and *mycC* (D). Dots were placed at locations with identical nucleotides when more than 45 of 60 nucleotides were identical. Amino acid adenylation domains of functional modules of synthetases are indicated by black boxes.

cantly different from that of strain 168 (data not shown), suggesting that the entire genomic structure of RB14 has little similarity to that of strain 168, including the location of *xyjF* and/or *xynD*.

However, unexpectedly, as shown in Fig. 5, the promoter region of the iturin A operon has significant homology to the

upstream sequence of *xynD* of strain 168. Since the downstream region of the iturin A operon has two tandem repeat DNA segments with significant homology to the *xynD* region of strain 168, it is highly probable that the coding region of the iturin A operon is transferred into the promoter of *xynD* and resides in this promoter, rather than that the promoter of *xynD* is simply transferred into the iturin A operon. The possibility that such a large antibiotic gene transfer occurs was also suggested for the tyrocidine operon, in which some peptide synthetase genes are integrated into the parental gramicidin S operon (21). In terms of horizontal transfer, it is possible that large DNA transfers occur frequently. The duplicated copy of *xynD* may indicate transient existence of a tandem repeat of early iturin A operons that were intermediates in the adenylation domain swapping mentioned above.

Our attempt to introduce a *repU* promoter instead of an intrinsic promoter into RB14 resulted in increased production of iturin A (Fig. 6), and the advantage of using the $P_{repU-neo}$ cassette was identified. This method might be useful for generating bacteria that act as effective biological control agents.

At present, we are able to compare very similar operons, such as surfactin operons of *B. subtilis* (3) and two lichenysin operons of *Bacillus licheniformis* (13, 45), plipastatin (37, 38)

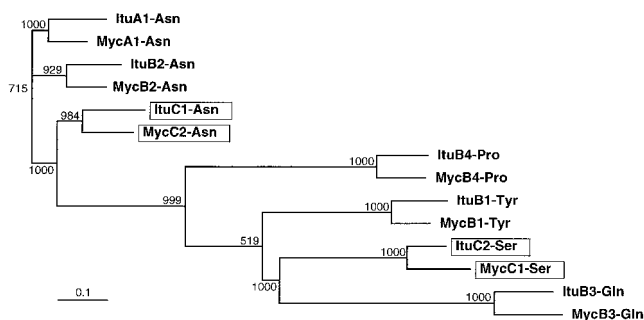


FIG. 8. Phylogenetic tree constructed by the neighbor-joining method based on the sequences of the adenylation domains (from part of the A3 motif, PKG to the A6 motif, GELC[Y]) of iturin A synthetase and mycosubtilin synthetase. The numbers are bootstrap values based on 1,000 replicates.

and fengycin (16) operons, and iturin A and mycosubtilin (4) operons. These comparisons may provide good techniques for elucidating the boundary of the functional domain that directs engineered peptide synthesis (22, 31, 33, 34).

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