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Construction and Physiological Studies on a Stable Bioengineered Strain of Shengjimycin

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Shengjimycin is a group of 4″-acylated spiramycins with 4″-isovalerylspiramycin as the major component, produced by recombinant *S. spiramyceticus* F21 harboring a 4″-*O*-acyltransferase gene from *S. mycarofaciens* 1748. A stable bioengineered strain of *Streptomyces spiramyceticus* WSJ-1 was constructed by integrating the 4″-*O*-acyltransferase gene (*ist*) by homologous recombination into the chromosome of the spiramycin-producing strain *S. spiramyceticus* F21. In this construction, a *Streptomyces/E. coli* shuttle plasmid pKC1139 (Am^R) was used as the vector with the *tsr* gene used as selection marker for homologous recombination. The constructed strain, *S. spiramyceticus* WSJ-1,was genetically stable in production titer and proportion of components of shengjimycin as well as in maintaining the *tsr* selective marker when grown without selection. Southern hybridization confirmed the integrated status of the *ist* gene in the host genome. The production and the proportion of major component of 4″-isovalerylspiramycin of *S. spiramyceticus* WSJ-1 was also improved comparing with the strain harboring an autonomous plasmid -*S. spiramyceticus* F21/pIJ680(311) as shown by HPLC analysis. Physiological studies indicated that increase of the VDH (valine dehydrogenase) and LDH (leucine dehydrogenase) activities of WSJ-1 may be involved in this improvement.

Genetically engineered strains of modified 16-membered macrolides have been reported and some have been useful to produce antibiotics with improved biological properties compared to the original ones[1~6]. Shengjimycin, a complex 4″-acyl- modified spiramycin with 4″-isovalerylspiramycin as major component, was produced by a genetically engineered strain *S. spiramyceticus* F21/pIJ680(311)[7]. The 4″-*O*-acyltransferase gene was cloned from *S. mycarofaciens* 1748[8], and confirmed to be identical to *car*E (unpublished data)†. Manufacturing of genetically engineered antibiotics requires stability in production and proportion of different components in complex antibiotics. The integration of heterologous genes into the chromosome usually will stabilize expression[9]. This paper describes the construction of a genetically stable strain for shengjimycin production by homologous recombination and studies on its physiological properties.

Materials and Methods

Medium

Slant, seed culture, fermentation and bioassay medium were prepared according to reference[8]. For physiological studies soluble fermentation medium was used in which starch and fishmeal were substituted with soluble starch and fishmeal extract. Media for protoplast preparation and regeneration of *Streptomyces* strains were prepared according to reference[10].

Strains and plasmids used in this work are listed in

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† Note: In shotgun cloning from the *Streptomyces mycarofaciens* 1748 genome we have found that there exist two genes related to 4′-acylase. The expression of one 4″-acylase gene (*ist*) in a spiramycin-producing strain resulted mainly in the production of 4″-isovalerylspiramycin. Another 4″-acylase gene (*mpt*) was related to production of 4″-propionylspiramycin. Nucleotide sequence analysis revealed that the ORF of *ist* gene starts at ATG and ends at TAG and is predicted to encode a protein of 388 amino acids. The coding sequence of *ist* was identical with that of CarE reported by EPP *et al.* while the *mpt* gene (accession number D63662) showed 67.9% identity with *car*E.

Table 1. Strains and plasmids used in this work.

Plasmid or strain	Characteristics[a]	Reference
Plasmid	Plasmid	This lab
pWHMI	pWHM3,harboring *Ist* gene,*bla*	
pKC1139	*E.coli/str.*shuttle vector,rep^{ts},Am^{R}	[11]
pCN3H8	cosmid clone containing PKSII homologous regions from *S.spiramyceticus* F21 genome	[12]
pNJ1	*E.coli-Str.*shuttle cosmid vector,*bla,tsr*	[13]
pSK(-)	pBluesript (-) vector,*bla*	[14]
pKB2	pKC1139 with *ist* insert	This work
pKB2VI	*Eco*RI-*Eco*RV1.0kb from pCN3H8 cloned in pKB2 *Eco*RI-*Eco*RV sites	This work
pSW1	*tsr* from pNJ1 cloned in pSK(-) *Bam*H1-*Pst*1 sites	This work
pSW2	*tsr* from pSW1 cloned in pKB2VI *Hind*III-*Xba*1 sites	This work
pSW4	*Hind*III-*Bgl*II 2.0kb from pCN3H8 cloned in pSW2 *Hind*III-*Bgl*II segment	This work
Strain		
E.coli DH 5α	*E.coli* cloning host	[14]
S.spiramyceticus F21	spiramycin producing-strain	This lab
Sarcina lutea	bioassay test organism	This lab
S.spiramyceticus F21/pIJ680(311)	*S.spiramyceticus* with *ist* gene in the autonomous form	[8]
S.spiramyceticus WSJ-1	*S.spiramyceticus* with *ist* gene in the integrated form	This work

[a]*bla*, ampicillin resistance gene; *tsr*, thiostrepton (Thio)resistance gene: rep^{ts}, temperature sensitive replication origin; Am^{R}, apramycin(Am) resistance gene; PKS, polyketide synthase genes .

Table 1.

Methods

DNA Manipulations

Bacterial culture and DNA manipulation were carried out as described by MANIATIS *et al*[14]. DNA recovery was followed according to the direction of USA Bio101 Co. Geneclean^{R} directions. *Streptomyces* culture, protoplast formation, regeneration and DNA transformation were carried out according to HOPWOOD *et al.* manual[9]. Southern hybridization was performed as described by Boeheringer Mannheim Dig DNA Labeling and Detection Kit instructions; the hybridization was performed at 55°C and washing was carried out at 68°C in 0.1×SSC twice.

Fermentation, Bioassay and TLC Analysis

For the fermentation of *S. spiramyceticus* WSJ-1 and F21/pIJ680(311), the titer and TLC analysis were performed as described previously[8]. Fermentation was repeated three times with 3 flasks in parallel.

Mycelial Growth

Mycelial growth was estimated as precipitated mycelium volume *versus* supernatant after centrifuging the seed culture broth at 3,000 rpm for 20 minutes, or was determined by measuring the cell dry weight from 2ml of fermentation broth.

Genetic Stability Test

S. spiramyceticus WSJ-1 and F21/pIJ680(311) were passed for successive generations without thiostrepton selection. The mycelia were sonicated (Cole Parmer Co. Ltd., USA) at 20% amplitude with 8-second pulses with 2-second interval for 2 minutes on ice, then diluted and plated on agar plates with and without antibiotic (25 μg/ml). After incubation for 5~7 day at 28°C, the colony count of the two plates were compared.

HPLC Analysis

The pH of fermentation broth was adjusted to 4.0 by adding formic acid, then adjusted to pH 8.5~9.0 with 1 M NaOH after filtration. The filtrate was extracted with ethyl acetate, and the organic phase was mixed with phosphate buffer at pH 2.0~2.5. The ethyl acetate was removed by evaporation after adjusting the pH of aqueous phase to 4.5~5.0. The aqueous phase was extracted again with ethyl acetate at pH 8.5~9.0. The extract was dried and dissolved in 50% of methanol. HPLC analysis was performed using a Schimadzu, LC-6A Scim-pack CLC-ODS, 60×150 mm column, methanol-1% NaH_2PO_4 (53 : 47); UV at 210 nm with a flow rate of 1 ml/minute.

VDH and LDH Assay

Valine and leucine dehydrogenase activities were measured as described[15]. Cell-free extract protein was prepared by growing cultures in soluble fermentation medium and the mycelium was disrupted by sonication for 5 minutes in ice following centrifugation at 4°C, 12,000 *g* for 15 minutes. Protein was measured according to LOWRY[16]. This experiment was repeated twice in three flasks parallel.

Results and Discussion

Construction of the Integrated Plasmid for Homologous Recombination

The construction of integrated recombinant plasmid with the *ist* gene employed a temperature sensitive derivative of pKC1139, derived from pSG_5[10]. The *ist* gene was obtained from pWHM1 and pKB2 was obtained by cloning the *ist* gene into plasmid pKC1139. For homologous recombination the PKSII homologous fragments of a cosmid pCN3H8 from a *S. spiramyceticus* F21 genomic library[11], which did not contain the coding genes for spiramycin biosynthesis, were chosen to ensure against any possible influence on spiramycin production when integration occured. Recombinant pKB2V1 was composed of pKB2 by cloning the *Eco*RI-*Eco*RV 1.0 kb fragment of pCH3H8 into the same sites. The *tsr* gene used as selection marker for integration,was obtained on the *Pst*I-*Bgl*II 1.2 kb fragment of plasmid pNJ1, after cloning into pSK(−), then cut with *Hind*III-*Xba*I and cloned into the corresponding sites of pKB2V1, resulting in the formation of pSW2. Plasmid pSW4 was obtained by the cloning of *Hind*III-*Bgl*II 2.0 kb fragment from pCN3H8 into the corresponding segment sites in pSW2 for generating the flank region in homologous recombination. The detailed scheme of construction was shown in Fig. 1.

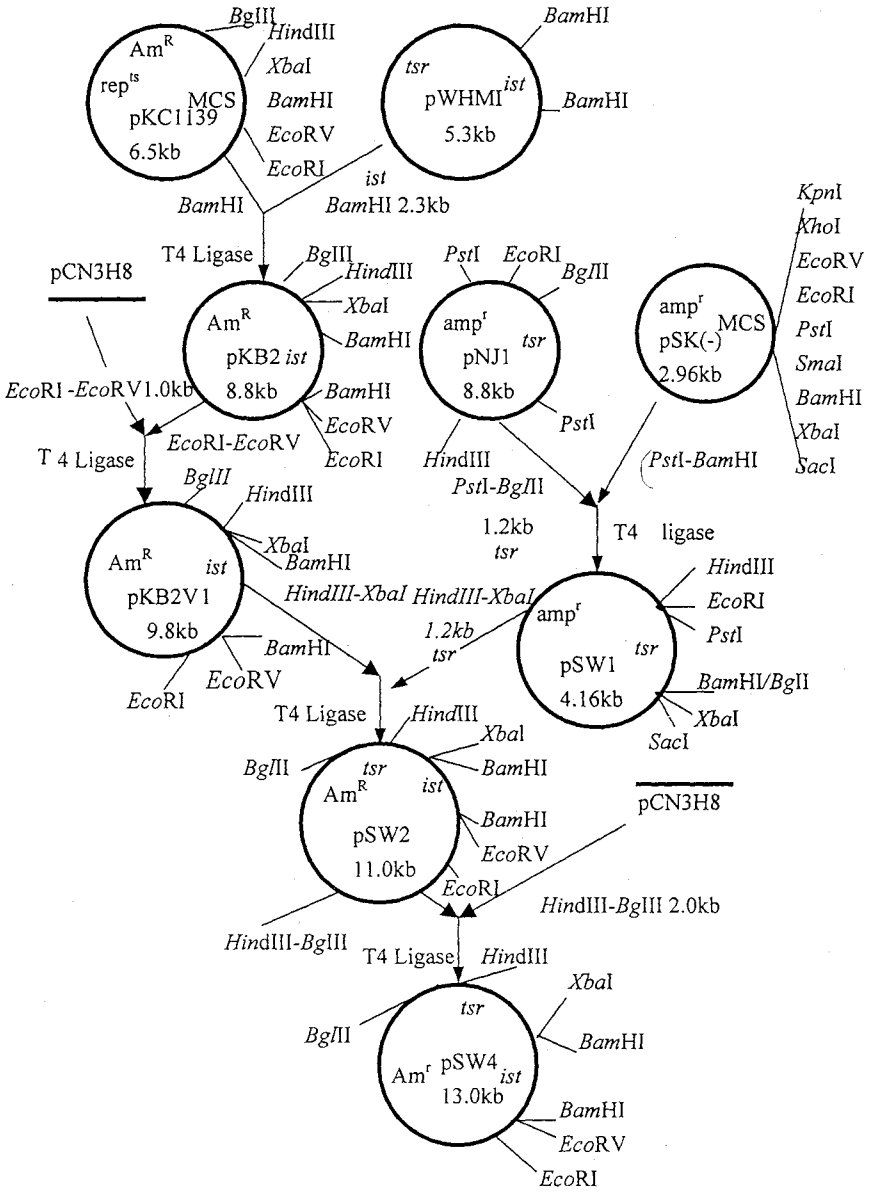
Screening for Stable, Integrated *ist* Recombinants

Transformants were obtained by introducing the pSW4 plasmid into protoplasts of *S. spiramyceticus* F21 mediated by 25% PEG. Stable transformant was purified by repeated screening at elevated temperature (37°C) on agar medium containing thiostrepton (25 μg/ml). *S. spiramyceticus* WSJ-1 strain was obtained, efficiency of plating on thiostrepton containing plate was 100 fold (800 : 7) higher than that on apramycin (50 μg/ml), showing that the *ist* gene would be integrated into *S. spiramyceticus* WSJ-1 by double crossover recombination events at *PKSII* region with plasmid-encoded *ist* occuring at low frequency. It has been reported[11], that DNA inserts are not 100% integrated when temperature sensitive plasmids (pSG_5 series) following homologous recombination.

Sourthern Hybridization

The total DNA of *S. spiramyceticus* WSJ-1, *S. spiramyceticus* F21/pIJ680(311) and *S. spiramyceticus* F21 as the control were digested with *Bam*HI or *Pst*I and

Fig. 1. Construction of integrated plasmid pSW4 for homologous recombination.



hybridized with the 2.3 kb *ist* gene. The result is shown in Fig. 2. No signal appeared in total DNA of the *S. spiramyceticus* F21 control. By contrast, the DNA of WSJ-1 gave a positive signal, a 2.3 kb band appeared when WSJ-1 and *S. spiramyceticus* F21/pIJ680 (311) DNA were digested with *Bam*HI and the 1.9 kb and 6.6 kb bands were appeared when *S. spiramyceticus* F21/pIJ680(311) DNA was digested with *Pst*I. A 7.0 kb band, in addition to the 1.9 kb and 6.6 kb bands, the fragments of pSW4 digested with *Pst*I, was clearly seen in lane 6 of WSJ-1 DNA digested with *Pst*I, indicating the integration of *ist* gene in the chromosome.

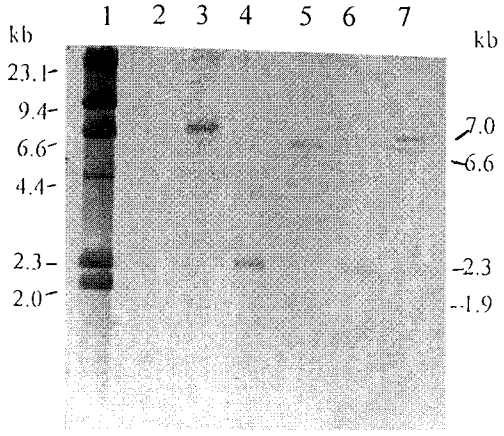
Fermentation Stability of *S. spiramyceticus* WSJ-1

S. spiramyceticus WSJ-1 was grown for five generations without selection, and the mycelial growth and production titer were measured. The components of fermentation product, shengjimycin, were assayed by TLC at the same time. The fermentation results are shown in Table 2 showing that fermentation of *S. Spiramyceticus* WSJ-1 was stable through several passages without selection. The

Table 2. Growth and production titer of *S. spiramyceticus* WSJ-1.

No. of passages	Growth of mycelium(%)	Production titer (μg/ml)
F1	33	823
F2	35	950
F3	37	835
F4	28	803
F5	33	780
x ± SD	33.2 ± 3.35	838 ± 65.9
S.spiramyceticus F21/pIJ680(311)		450 ± 70.8

Fig. 2. Analysis of the chromosomal DNA of strain WSJ-1 by Southern hybridization using *ist* gene as a probe.



1: λDNA/*Hind*III, 2: DNA of F21, 3: DNA of WSJ-1, 4: DNA of F21/pIJ680(311)/*Bam*HI, 5: DNA of F21/pIJ680(311)/*Pst*1, 6: DNA of WSJ-1/*Bam*HI, 7: DNA of WSJ-1/*Pst*1

composition of fermentation products was also stable (TLC data not shown). In comparison with *S. spiramyceticus* F21/pIJ680(311), the production titer of WSJ-1 was greatly improved.

Genetic Stability of WSJ-1 Strain

S. spiramyceticus WSJ-1 was grown for five generations without selection, and the colony count on agar with or without thiostrepton (Thio) was determined. As shown in Table 3, the numbers of colonies of *S. spiramyceticus* WSJ-1 on both plates were essentially equal, but *S. spiramyceticus* F21/pIJ680(311) showed plasmid loss.

Table 3. Thiostrepton (Thio) -resistance maintenance after successive passages of *S. spiramyceticus* WSJ-1.

No.of passages	Thio(+)	Thio(-)
F1	760	740
F2	188	211
F3	101	91
F4	560	570
F5	440	470
x ± SD	409 ± 269	416 ± 264
S.spiramyceticus F21/pIJ680(311)	201	560

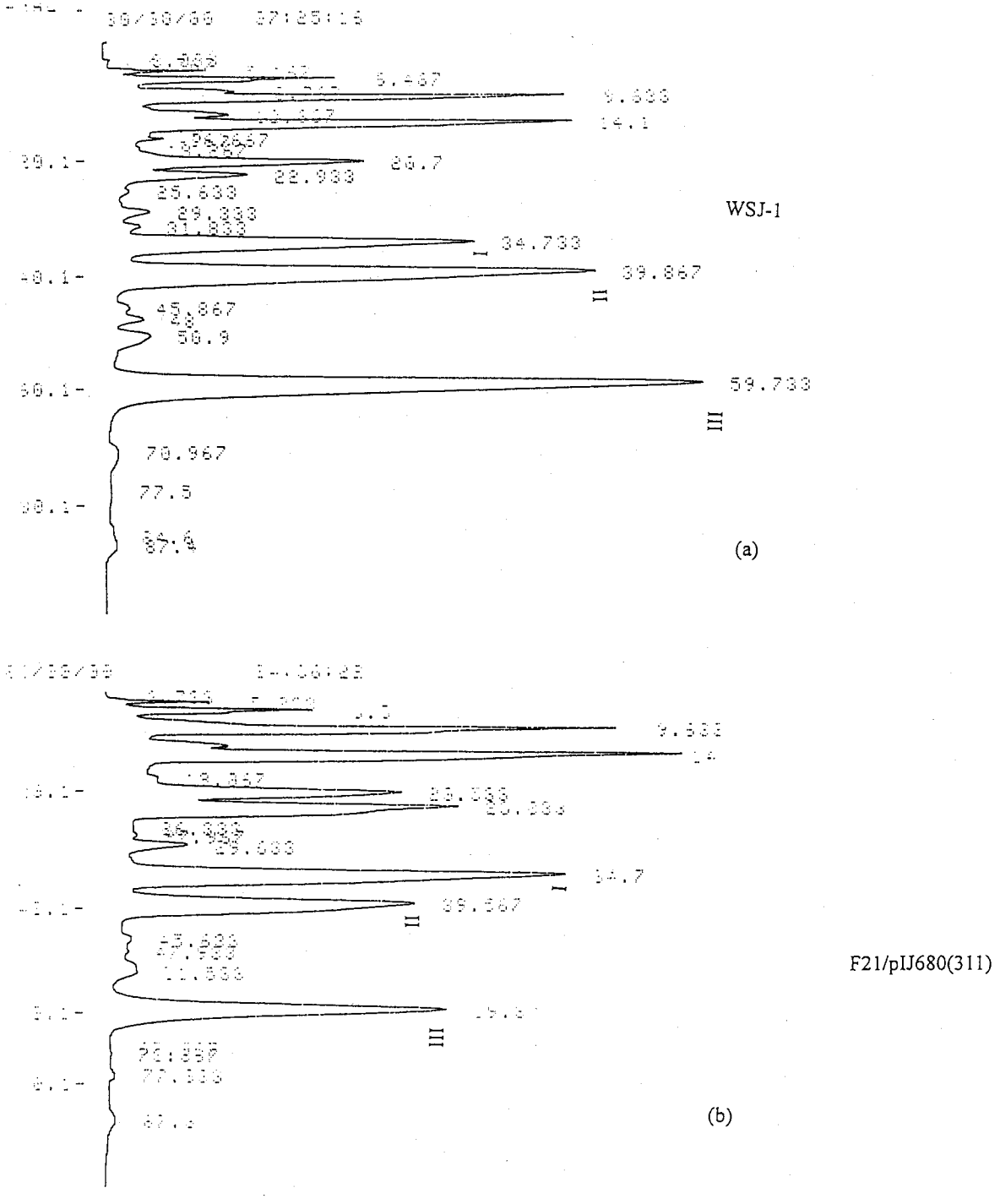
HPLC Analysis of Shengjimycin Components of WSJ-1

To examine the shengjimycin components produced by WSJ-1, the strains WSJ-1 and F21/pIJ680(311) were grown for 4 days in fermentation medium in eight batches and the products were compared by HPLC (Fig. 3). The recombinant *S. spiramyceticus* F21 produced various 4″-acyl spiramycin and isovalerylspiramycin I,II,III as major compound with RTs of 34.73, 39.867 and 58.733 minutes, respectively. 55.2% isovalerylspiramycin was produced by WSJ-1 and, in contrast, 39.5% was produced by F21/pIJ680 (311) strain, suggesting the proportion of major components produced by the genetically stable strain was signicantly improved.

Growth and Shengjimycin Production Time Course of WSJ-1

In order to investigate physiological differences between WSJ-1 and F21/pIJ680(311), time course of growth, fermentation and productivity studies were carried out as summarized in Table 4.

Fig. 3. HPLC of shengjimycin produced by (a) WSJ-1 and (b) F21/pIJ680(311).



It was observed that the mycelial growth of both starins was about the same in batch fermentation, but the fermentation titer and productivity of WSJ-1 was much higher than the F21/pIJ680(311), indicating a significant difference in antibiotic production of these two strains.

The VDH and LDH Specific Activity

To identify physiological differences between WSJ-1 and F21/pIJ680 (311) strains, VDH and LDH specific activities of both strains were measured. The specific activities of VDH and LDH during shengjimycin fermentation are

Table 4. Growth and shengjimycin production time course in batch fermentation.

Time (h)	Dry cell weight (mg/ml)		Production ($\mu g/ml$)		Productivity ($\mu g/mg$)	
	WSJ-1	F21/pIJ680 (311)	WSJ-1	F21/pIJ680(311)	WSJ-1	F21/pIJ680(311)
24	21.75	27.69	195	58	8.96	2.09
48	26.97	30.01	498	148	18.46	4.93
72	29.73	29.06	1000	408	33.6	14.0
96	32.52	19.93	1114	280	34.2	14.0
120	25.81	22.73	944	199	36.6	8.75

Fig. 4. The course of VDH specific activity of WSJ-1 and F21/pIJ680(311).

—◆—: F21/pIJ680(311), —■—: WSJ-1

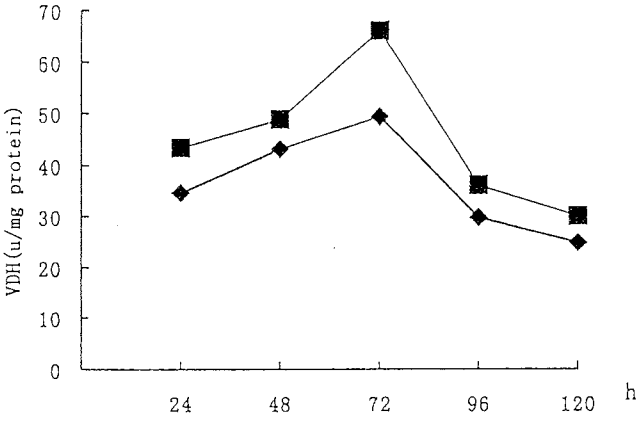
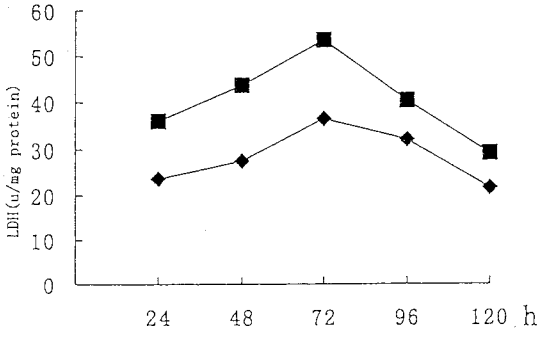


Fig. 5. The course of LDH specific activity of WSJ-1 and F21/pIJ680(311).

—◆—: F21/pIJ680(311), —■—: WSJ-1



shown in Fig. 4 and 5. The VDH and LDH specific activities of WSJ-1 reached 55.92 and 53.37 u/mg protein at 72 hours whereas the maximum was about 29.77 and 36.36 u/mg with F21/pIJ680 (311), respectively.

It is known that valine catabolism supplies precursors for macrolide-lactone formation and the level of VDH favors spiramycin production as the substrate of isovaleryl-transferase. However L-leucine is a precursor of isovaleryl-CoA, which, as noted elsewhere, is usually present in low concentration in *Streptomyces* fermentation[17]. Increase of LDH activity favors isovaleryl-CoA production, As a consequence, an increase of VDH and LDH specific activities of WSJ-1 could result in increased accumulation of spiramycin and isovaleryl CoA which may facilitate the formation of isovalerylspiramycin. The reason for the stimulation of VDH and LDH activities in WSJ-1 needs to be investigated further.

The practical application of genetically-engineering of hybrid antibiotic production presents technical problems. A growing body of evidence indicates that the introduction and maintenance of foreign genes in self-replicating plasmids may frequently cause substantial reductions in secondary metabolite yields[18]. Maintaining cloned genes in recombinant Actinomycetes in absence of selection sometimes is required. The issue of catabolism regulation associated providing precursors for antibiotic biosynthesis poses another obstacle. Here, we have presented an example of the construction of a stable genetically engineered strain with improved antibiotic production capability.

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