Sequence completion, identification and definition of the fengycin operon in *Bacillus subtilis* 168

Valentina Tosato,¹ Alessandra M. Albertini,² Michela Zotti,¹ Sabrina Sonda¹ and Carlo V. Bruschi¹

Author for correspondence: Carlo V. Bruschi. Tel: +39 40 375 7304. Fax: +39 40 375 7343.
e-mail: bruschi@icgeb.trieste.it

¹ Microbiology Group, International Centre for Genetic Engineering and Biotechnology and ITALTBS Ricerche, Srl, AREA Science Park, Padriciano 99, I-34012 Trieste, Italy

² Department of Genetics and Microbiology 'A. Buzzati Traverso', University of Pavia, 27100 Pavia, Italy

A 15 kb DNA fragment from the *Bacillus subtilis* chromosome between *citB* and *ppsC* has been sequenced, and new ORFs encoding putative enzymes involved in lipopolypeptide synthesis, which complete a partial operon previously reported, and a new set of enzymes responsible for lipid metabolism have been identified. From the analysis of DNA sequence homology of the fragment it was deduced that these new peptide synthetase genes are part of an operon for the biosynthesis of the fungicide fengycin.

Keywords: antifungal antibiotic, *Bacillus subtilis*, fengycin, lipopeptide synthetase

INTRODUCTION

Of the secondary metabolites of biotechnological relevance produced by *Bacillus subtilis*, lipopolypeptides with antimicrobial activity are among the most important. These small molecules, some of which, such as gramicidin, iturins and surfactins, are well known (Tomino *et al.*, 1967; Maget-Dana & Peypoux, 1994; Ullrich *et al.*, 1991), are synthesized nonribosomally, through a thioester template mechanism, by synthetases that show a highly conserved structural organization into specific peptide-binding domains (Kleinkauf & von Döhren, 1990; Turgay *et al.*, 1992; Stachelhaus *et al.*, 1995).

Through the systematic DNA sequencing of the chromosome of *B. subtilis* strain 168, the initial characterization of part of a large multienzyme complex putatively involved in the biosynthesis of a new lipopolypeptide has been described (Tognoni *et al.*, 1995). This region comprised three genes: *pps orf1*, *pps orf2* and, partially, *pps orf3*. A corresponding transcriptional unit has been genetically dissected by transposon mutagenesis in strain F29-3 (Chen *et al.*, 1995) and found to encode the multienzyme complex responsible for the biosynthesis of fengycin, the amino acid composition of

which was studied by Vanittanakom & Loeffler (1986). While in the latter strain fengycin production can be demonstrated in a specific bioassay, in strain 168 production has not yet been proven.

In the present paper, we report the completion of the DNA sequencing of the genes specifying the entire polypeptide complex and the analysis of its structural organization. The 3'-end of a fourth ORF has been found together with a complete ORF encoding a fifth putative enzyme, which is similar to the *fenB* gene product sequenced by Chen *et al.* (1995). A transcription termination signal is located immediately downstream of the putative operon. The portion of the chromosomal sequence between the truncated *pps orf3* and the last part of the fourth gene that we characterized was obtained from the Pasteur database of the *B. subtilis* DNA sequence. Based upon the analysis of all the ORFs, we concluded that this five-gene operon encodes a multienzyme complex similar to that responsible for the biosynthesis of fengycin in strain F29-3. Moreover, on the 3'-side of the fifth gene we discovered six ORFs encoding enzymes putatively involved in fatty acid metabolism.

METHODS

Bacterial strains, plasmids and culture conditions. *B. subtilis* 168 *trpC2* from Institut Pasteur (Paris, France) was used as the source of chromosomal DNA for PCR amplifications of the

The EMBL accession number for the sequence reported in this paper is Y13917.

Table 1. Plasmids used in this study

Plasmid	Description (source or reference)
pBSKII/pBKSII	Ap; integrative vector (Short <i>et al.</i> , 1988)
pUC18	Ap; integrative vector (Yanisch-Perron <i>et al.</i> , 1985)
pDIA5304	Ap Cm; vector used for chromosome walking (Glaser <i>et al.</i> , 1993)
pCE18	15.3 kb in pDIA5304 obtained by chromosome walking (Glaser <i>et al.</i> , 1993)
pCE19	8.3 kb <i>Bam</i> HI– <i>Sac</i> I chromosomal fragment cloned into pBKSII
pCE20	7.0 kb <i>Bam</i> HI– <i>Xho</i> I fragment from pCE18 cloned into pBKSII
pVA1	3.6 kb <i>Sac</i> I– <i>Hind</i> III fragment from pCE19 cloned in pBKSII
pVA2	2.4 kb <i>Hind</i> III fragment from pCE19 cloned in pBKSII
pVA3	1.5 kb <i>Hind</i> III fragment from pCE19 cloned in pBKSII
pVA4	0.4 kb <i>Hind</i> III fragment from pCE19 cloned in pBKSII
pVA5	0.3 kb <i>Hind</i> III fragment from pCE19 cloned in pBKSII
pVL1	1.7 kb <i>Dra</i> I– <i>Sac</i> I fragment from pVA1 cloned in pBKSII
pVL2	1.2 kb <i>Dra</i> I fragment from pVA1 cloned in pBKSII
pVL3	0.8 kb <i>Dra</i> I– <i>Hind</i> III fragment from pVA1 cloned in pBKSII
pVE1	0.3 kb <i>Dra</i> I– <i>Hind</i> III fragment from pVA2 cloned in pBKSII
pVE2	1.4 kb <i>Dra</i> I fragment from pVA2 cloned in pBKSII
pVE3	0.5 kb <i>Dra</i> I– <i>Hind</i> III fragment from pVA2 cloned in pBKSII
pVN1	2.6 kb <i>Pst</i> I fragment from pCE20 cloned in pBKSII
pVN2	1.1 kb <i>Pst</i> I fragment from pCE20 cloned in pBKSII
pVN3	1.0 kb <i>Pst</i> I fragment from pCE20 cloned in pBKSII
pVN4	1.8 kb <i>Bam</i> HI– <i>Pst</i> I fragment from pCE20 cloned in pBKSII
pVT1	0.6 kb <i>Bam</i> HI fragment obtained by PCR amplification on chromosomal DNA and cloned in pBKSII
pVI1	0.3 kb fragment obtained by PCR amplification on chromosomal DNA, blunt-ended by Klenow enzyme and cloned in pUC18

overlapping fragments to be cloned and sequenced. The chromosomal DNA was extracted from overnight cultures as described by Murray & Thompson (1980).

Escherichia coli DH5 α -(*supE44* Δ *lacU169* ϕ 80 *lacZAM15* *hsdR17* *recA1* *gyrA96* *thi-1* *relA1* F[–]) (Gibco-BRL)-competent cells were transformed with the recombinant plasmid pCE19. *E. coli* and *B. subtilis* strains were grown aerobically at 37 °C in Luria–Bertani or Terrific Broth. When necessary, these were supplemented with 100 μ g ampicillin (Ap) ml^{–1}.

If not indicated otherwise, *E. coli* XL-1 Blue MRF' [Δ (*mcrA*)183 Δ (*mcrCB*–*hsdSMR*–*mrr*)173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* (F' *proAB*⁺ *lacI*^a *lacZAM15*-Tn10-[Tet^r]); Stratagene] was used as the recipient strain for cloning experiments.

The plasmids used in this work are listed in Table 1.

Transformation and DNA manipulation. *E. coli* cells were transformed following the procedure of Hanahan (1985).

Plasmid DNA was isolated from *E. coli* by a modified alkaline lysis procedure (Zhou *et al.*, 1990) and all DNA manipulations were performed as described by Sambrook *et al.* (1989). Double-stranded DNA templates for sequencing reactions were purified by MicroSpin S-400 HR columns (Pharmacia).

PCR amplification. To complete the contig next to the *ppsD* gene, a PCR amplification was performed on *B. subtilis* 168 genomic DNA using custom primers with a *Bam*HI recognition site at their end. The forward primer (PF Bam) was a 32-mer with the sequence 5'-CGGGATCCCCG-

TCAAAGCGTTACAGGTATCTGC-3' (the underlined bases correspond to the end of *ppsD*). The reverse primer (PB2 Bam) was a 31-mer with the sequence 5'-CGGGATCCCGTACCCTAAAGCCAAATCTTCA-3' (the underlined bases correspond to the end of *ppsE*). PCR was run for 30 cycles in a Prem-ThermoCycler (Lep Scientific) apparatus using the following protocol: denaturation, 3 min at 94 °C for the first cycle and 1 min at 94 °C thereafter; annealing, 1 min at 57 °C; elongation, 1 min at 72 °C. The PCR product was purified by electroelution, then digested with *Bam*HI and cloned into pBSKII (Stratagene). The junction between 7 kb and 8.3 kb contigs was verified by sequencing a PCR product of about 300 bp obtained with the 22-mer forward primer PD2 (5'-TCGATGCTGTTCATATGGATGA-3'), a custom primer also used for the 7 kb fragment sequencing, and the 22-mer reverse primer PR1 (5'-TTTTGACTTCATTGCTCCACCA-3'), designed at the end of the 8.3 kb sequence. The 300 bp purified PCR product was cloned by the SureClone Ligation kit into a pUC18 *Sma*I/BAP vector (Pharmacia).

DNA cloning and sequencing. We received a fragment of approximately 15.3 kb cloned into a *Sac*I/*Sma*I-digested pDIA5304 vector (Glaser *et al.*, 1993) from the laboratory of Dr A. Galizzi, University of Pavia, Italy. This recombinant plasmid was recovered by a Campbell-type recombination event and it contains a fragment adjacent to an already known chromosomal marker (Niaudet *et al.*, 1982). The recombinant plasmid was cut with *Bam*HI and *Xho*I and the derived 7 kb fragment was cloned into a pBSKII vector. The fragment was cleaved with *Pst*I into four pieces (about 2.7 kb, 1.8 kb and a doublet of 1.0 kb). The remaining 8.3 kb was obtained from

the above laboratory as a subclone in a *Bam*HI–*Sac*I-cleaved pBSKII vector. The fragment was subcloned using *Hind*III and the resulting five fragments (3.6, 2.4, 1.5, 0.4 and 0.3 kb, respectively) were inserted in pBSKII. The 3.6 and 2.4 kb fragments were furthermore cut with *Dra*I and subcloned into blunt-ended *Eco*RV-digested pBSKII.

All subclones were sequenced on both strands using primer walking by the dideoxy chain-termination method (Sanger *et al.*, 1977) with plasmid double-stranded DNA as template and a Sequenase version 2.0 sequencing kit (USB). For the sequencing reactions we used M13 universal primers (Stratagene).

Data handling and computer analysis. Autoradiographic films were read manually on an IBI gel reader using the MacVector sequence analysis program (Eastman Kodak). Sequence alignments and verification of the overlaps were performed using the AssemblyLign software package (Eastman Kodak). Preliminary searches for putative ORFs were performed with the IBI/Pustell sequence analysis program (Pustell & Kafatos, 1984). Final analysis of contiguous DNA sequences and possible translations into proteins were obtained using the GCG software package (Devereux *et al.*, 1984). Amino acid sequences, ORFs and restriction maps were calculated with both the commercial DNA Strider software (Marck, 1988) and the ORFEX program developed in our laboratory (P. Zaccaria & C. Bruschi, unpublished results). Codon usage and possible frameshift analyses were performed by the GeneMark e-mail server (Borodovsky *et al.*, 1994). The program DNAsis (Pharmacia) was used to design the primers for sequencing. Sequences were compared to those in the GenBank, SBASE (Pongor *et al.*, 1993) and EMBL databases by the BLAST e-mail server (Altschul *et al.*, 1990). In particular, we used BLASTX, BLASTN and BLASTP for homology searches of nucleotide and amino acid sequences, respectively. Protein homology searches were carried out by FASTA (Pearson & Lipman, 1988) against the SWISS-PROT, NBRF-PIR and GenPep databases.

RESULTS

Sequence determination and analysis

As part of the European Community Biotechnology programme for the systematic sequencing of the *B. subtilis* 168 genome, we were assigned a region located at about 167° on the circular chromosome which is flanked by the genes *citB* and *ppsC*. Fifteen kilobases (consisting of two contigs of 8.3 and 7 kb) were fragmented, subcloned and sequenced as described in

Methods. A preliminary restriction map was initially made on the plasmid clones pCE19 (containing the 8.3 kb fragment) and pCE20 (containing the 7.0 kb fragment) to find fragments of suitable size for sequencing. Eleven subclones from the fragmented 8.3 kb contig and four subclones from the 7 kb contig were sequenced and these could be located on the basis of the preliminary restriction map. The restriction enzyme *Sfi*I does not cleave in this region, but a *Not*I site was found at 1267 bp (Fig. 1) from the 5′-end of the 7 kb contig according to the physical map of the chromosome (Itaya & Tanaka, 1991). The junctions between the restriction fragments were sequenced using pCE19 and pCE20 as templates. A fragment of 131 nucleotides, derived from the *Pst*I-digested 7 kb contig, was directly sequenced on pCE20. Because of the difficulties encountered at the level of pCE18 template preparation due to its size (about 20 kb), the sequence of the junction between the 8 and 7 kb contigs was verified by PCR. A product of 413 bp containing the junction was amplified using two primers designed on the two contigs, cloned and sequenced. At the 3′-end of the 8 kb fragment, following the *Sac*I site, we found the overlap with the next contig assigned to Karl Entian's group (Frankfurt, Germany). However, when we compared the 5′-end of the 7 kb sequence with the sequences already deposited at the Pasteur database by the ENIRICERCHE group (G. Grandi, Milan, Italy), we could not find the expected overlap. Therefore, to cover the gap between the 7 kb fragment and the 3′-end of the fragment allocated to ENIRICERCHE, we designed a primer on each of the two sequences and amplified, cloned and sequenced a DNA fragment of 625 bp, 260 of which were new.

We sequenced 15276 bp entirely on both strands. A primer walking strategy allowed us to obtain a good level of accuracy of the sequence with a redundancy factor of 2.0. Since we verified that the systematic use of the dGTP analogue 7-deaza-dGTP did not solve the high number of compressions observed, the ambiguities were solved by using dITP in the reactions. The GC content was 45.3 mol %, which is slightly higher than the mean value for the *B. subtilis* genome. Since small genes are abundant in *B. subtilis* (Klier *et al.*, 1992), we decided to perform the sequence analysis using a minimum ORF length of 75 codons.

Fig. 1. Schematic representation of the chromosomal location of the 15 kb region with the relevant restriction sites: N, *Not*I; P, *Pst*I; B, *Bam*HI; H, *Hind*III; D, *Dra*I; S, *Sac*I. On the bottom, the arrows indicate the orientation of the 11 ORFs found.

Table 2. General features of the identified ORFs

ORF	Gene name	Endpoints (nt)	Translation start*	Stop codon
1	<i>ppsD</i>	1<-1601	-	TAA
2	<i>ppsE</i>	1633-5448	AGGTGcagacact ATG	TAA
3	<i>yosA</i>	5562-5951	GGAGGcttctca ATG	TAA
4	<i>yotA</i>	6090-7619	GGAGAAgaacaga ATG	TAA
5	<i>yotB</i>	7893-8195	GGAGATgct GTG	TGA
6	<i>yotC</i>	8289-9428	AAGGAGGaaacgat GTG	TAA
7	<i>yotD</i>	9552-11120	GAGATgctgtr GTG	TGA
8	<i>yotE</i>	11133-12464	GAGGTGaaatc ATG	TAA
9	<i>yotF</i>	12718-13614	GAGGAagacc ATG	TGA
10	<i>yotG</i>	13638-14417	GGAGGAcgcataca ATG	TAA
11	<i>yotH</i>	14437->15276	AGGGAGGcaatgctg ATG	-

* The Shine-Dalgarno sequence is in capital letters; the initiation codon is in bold.

Computer-assisted translation of the 15.3 kb DNA sequence in all six possible reading frames identified at least 40 putative ORFs (28 on the 8304 bp fragment and 12 on the 6978 bp fragment) among which, according to the three criteria proposed by Glaser *et al.* (1993), only 11 represented coding sequences (Fig. 1). The initiation codon appeared to be ATG in seven cases and GTG in three cases (*orf5*, *orf6* and *orf7*). The sizes and names of the genes, their Shine-Dalgarno consensus sequences and the start and stop codons are shown in Table 2. All putative genes are in the same orientation, opposite to the direction of movement of the replication fork.

Putative ORF products

Comparison of the amino acid sequences of each putative ORF product with those of proteins available in databases (Table 3) revealed identity of the polypeptide encoded by *orf1* with the carboxy-terminus of the *B. subtilis* peptide synthetase ORF4 (accession number Z34883). Analysis of the 533-aa-long polypeptide revealed significant homology with surfactin synthetase and gramicidin S synthetase. We therefore assigned the name *ppsD* to this truncated ORF. The deduced 1272 aa product of *orf2* showed 80% identity and 89% similarity over 1223 aa at the amino-terminus with the 1274 aa fengycin synthetase of *B. subtilis* F29-3 (Chen *et al.*, 1995). A CAA codon encoding a glutamine residue present in FenB at position 1224 is missing in *ppsE*. Therefore, the homology in the remaining carboxy-terminus portion decreases and the genes encoding the two peptides become significantly different (only 35% identity) in the last 40 nucleotides at the 5'-end and, consequently, in the corresponding amino acids. Comparison of our *orf2* sequence with a sequence deposited in the EMBL database (accession number Z34883) revealed 11 mismatches in 5496 bp of overlap. This results in the substitution of more than 100 aa in the corresponding protein and causes a decrease in homology with the fengycin synthetase described by Chen *et al.* (1995) (BLASTX scores: 5173 for our protein; 2773 for

protein Z34883). *orf1* and *orf2*, the latter now being called *ppsE*, seem to be part of an operon, which is followed by a 24 nucleotide palindromic sequence with putative transcription termination function, while no other termination sequences are present between the ORFs.

The functions of *orf3*, *orf4* and *orf5* are unknown. The putative products of the remaining ORFs in the 8 kb contig show significant homology with enzymes of fatty acid metabolism of *B. subtilis*. In particular, the product of *orf6* (*YotC*) shows similarities with acyl-CoA dehydrogenases of bacterial species (up to 52% identity with this enzyme from *Clostridium acetobutylicum*) and some mammalian species (up to 56% identity with the short-chain acyl-CoA dehydrogenase precursor in *Homo sapiens* and *Rattus norvegicus*). The deduced *orf7* product (*YotD*) shows similarities with a 4-coumarate-CoA ligase of plants (36, 35 and 33% identity with *Petroselinum crispum*, *Solanum tuberosum* and *Oryza sativa*, respectively). Furthermore, an interesting homology exists between this enzyme and the long-chain acyl-CoA synthetase of *B. subtilis* (accession number Z75208) and *E. coli* (accession number L17309). *orf8*, which forms an operon with the above-mentioned CoA ligase gene, encodes a putative biotin carboxylase (52% identity with the corresponding proteins of *Methanococcus jannaschii* and *B. subtilis*). This enzyme contains carbamoyl-phosphate synthetase subdomain signatures that are characteristic of several biotin-dependent enzymes, such as acetyl-CoA and propionyl-CoA carboxylases (Waldrop *et al.*, 1994). The last gene in this contig, *yotH*, which is truncated, shows homology with the amino-terminal part of a peptide similar to the beta chain of a prokaryotic biotin-dependent propionyl-CoA carboxylase. Therefore, these two enzymes could have related functions and are possibly involved in the first step of fatty acid biosynthesis. The putative protein encoded by *orf9* has 46-47% identity with the hydroxymethylglutaryl-CoA lyase precursor of mammalian species and it also shows good homology with the corresponding protein in

Table 3. ORF products showing similarities to known protein sequences

Product	Size (aa)	Similar protein(s) in database	Homology score	Percentage match (over aa)	Database accession no.	Motif
PpsD	533*	Peptide synthetase ORF4, <i>B. subtilis</i>	2766	100 (533)	Z34883	
		Peptide synthetase ORF3, <i>B. subtilis</i>	606	46 (233)	Z34883	
		Peptide synthetase ORF2, <i>B. subtilis</i>	292	48 (106)	Z34883	
		Surfactin synthetase, <i>B. subtilis</i>	473	42 (212)	P27206	
PpsE	1272	Fengycin synthetase, <i>B. subtilis</i>	5173	80 (1222)	L42523	ATP binding
		Peptide synthetase ORF5, <i>B. subtilis</i>	3722	99 (717)	Z34883	
		Gramicidin S synthetase II, <i>Bacillus brevis</i>	1246	47 (495)	D29676	Pantetheine 4'-phosphate site
		Surfactin synthetase, <i>B. subtilis</i>	887	57 (298)	Q04747	
YotA	510	f439; unknown function, <i>E. coli</i>	254	51 (82)	AE000246	
YotC	380	Acyl-CoA dehydrogenase (butyryl-CoA dehydrogenase), <i>C. acetobutylicum</i>	523	52 (186)	P52042	Acyl-CoA dehydrogenase I and II signatures
		Hepatic acyl-CoA dehydrogenase short-chain precursor, <i>R. norvegicus</i>	478	50 (199)	J05030	
		YqiN, <i>B. subtilis</i>	454	47 (182)	D84432	
YotD	523	Acyl-CoA synthetase long chain, <i>B. subtilis</i>	321	43 (139)	Z75208	ATP binding
		4-Coumarate-CoA ligase, <i>Nicotiana tabacum</i>	316	34 (187)	U50845	Prokaryotic membrane lipoprotein lipid attachment site
		4-Coumarate-CoA ligase, <i>P. crispum</i>	307	36 (175)	P14913	
		4-Coumarate-CoA ligase, <i>S. tuberosum</i>	307	33 (187)	P31684	
		4-Coumarate-CoA ligase, <i>O. sativa</i>	238	36 (122)	P17814	
YotE	444	Biotin carboxylase, <i>M. jannaschii</i>	776	52 (285)	U67563	Carbamoyl-phosphate synthase subdomain signatures
		YqhX, <i>B. subtilis</i>	787	52 (286)	D84432	
		Biotin carboxylase, <i>Synechococcus</i> PCC 7942	991	54 (347)	U59234	
		Biotin carboxylase, <i>B. subtilis</i>	773	52 (286)	P49787	
YotF	299	3-Hydroxy-3-methylglutaryl-CoA lyase, <i>Rhodospirillum rubrum</i>	675	46 (288)	U41280	
		3-Hydroxy-3-methylglutaryl-CoA lyase precursor, <i>H. sapiens</i>	673	46 (291)	P35914	
YotG	260	Enoyl-CoA hydratase, <i>H. sapiens</i>	388	46 (173)	X79888	Enoyl-CoA hydratase/isomerase signature
		3-Hydroxybutyryl-CoA dehydratase (crotonase), <i>C. acetobutylicum</i>	557	45 (245)	P52046	
		Enoyl-CoA hydratase, <i>R. norvegicus</i>	356	46 (165)	P14604	
YotH	280*	Methylmalonyl-CoA decarboxylase, <i>Veillonella parvula</i>	241	39 (114)	A49094	
		Propionyl-CoA carboxylase, <i>Mycobacterium leprae</i>	239	43 (117)	P53002	
		Propionyl-CoA carboxylase YqjD, <i>B. subtilis</i>	234	41 (117)	P54541	

* Truncated proteins.

Gram-positive and Gram-negative bacteria. Finally, the putative product of *orf10* is a polypeptide similar to various prokaryotic and eukaryotic enoyl-CoA hydratases. A summary of the most relevant BLASTX scores is shown in Table 3.

Motif searches revealed that PpsD and PpsE carry potential ATP- and 4'-phosphopantetheine-binding sites, typical of peptide synthetases. Such sites have

already been found in the other components of this multienzyme complex (Tognoni *et al.*, 1995). In particular, the consensus sequences SGT(S)TGKPRG, YGP(V/S)TE, NGK, defining the nucleotide-binding sites, were localized at the expected positions of each protein (Cosmina *et al.*, 1993). Based on the known distance in amino acids between two ATP- or phosphopantetheine-binding sites in the same protein (about 1035 aa), and the location of the highly conserved

sequence HHILDGW, it is possible to define the various protein domains. Furthermore, the sequence LGG(D/H)-S(I/L), which characterizes each amino-acid-binding site according to the description of the activating domains of gramicidin S synthetase (Schlumbohm *et al.*, 1991), is present at the carboxy-terminal region of each domain. PpsC (2555 aa) seems to be organized in two structural domains, like PpsA and PpsB described by Tognoni *et al.* (1995), while PpsD (3603 aa) and PpsE (1272 aa) have three domains and one domain, respectively.

DISCUSSION

Three peptide synthetase genes named *pps orf1*, *pps orf2* and *pps orf3* (partial) were described in previously published work (Tognoni *et al.*, 1995). The remaining portion of the third sequence and the partial sequence of a fourth gene (*pps orf4*) were available from the Pasteur database (accession number SL675-1). These data, combined with the data obtained in our laboratory, allowed us to construct the functional organization of a single operon. The last gene (*ppsE*) shares a high degree (90%) of identity with the *fenB* gene identified by Chen *et al.* (1995) as forming part of the fengycin synthetase operon. Therefore, it appears that *B. subtilis* strain 168 has a similar set of genes encoding enzymes involved in fengycin synthesis to strain F29-3, in disagreement with the results reported by Chen *et al.* (1995), in which the corresponding DNA hybridization between strains F29-3 and 168 could not be detected. Furthermore, downstream of the peptide synthetase genes we have identified five genes encoding enzymes probably involved in fatty acid metabolism, similar to what has been described by the above-mentioned authors. All the products of these genes show similarity with CoA enzymes involved in both biosynthesis and degradation of cellular lipids. Furthermore, YotD shares a region of sequence similarity with gramicidin, tyrocidine and L- α -aminoadipyl-L-cysteinyl-D-valine synthetases (the last one is responsible for the activation step in the biosynthesis of penicillin and cephalosporin). It is known that the multienzymic mechanism of nonribosomal peptide syn-

thesis is related to fatty acid synthesis (Zuber *et al.*, 1993), since it uses a pantetheine cofactor and one or more thioesterases (Krättschmar *et al.*, 1989). Therefore, the proximity of these genes to the peptide synthetase genes may suggest that their products are functionally related to the overall biosynthesis of the lipopeptide.

The Pps proteins form a multienzyme complex composed of ten structural domains, each characterized by several conserved regions and cofactor-binding signatures typical of enzymes that catalyse the biosynthesis of peptide antibiotics. Moreover, it has been reported that a correlation exists between the number and the order of structural domains and the distribution of the amino acids in the lipopeptide surfactin (Cosmina *et al.*, 1993). According to this view and further to our functional analysis conducted by homology searches in databases, we propose the following model for the organization of the fengycin lipopeptide (Fig. 2). Three out of ten amino-acid-binding structural domains have been identified by Tognoni *et al.* (1995), allowing the assignment of L-Glx to the first and fifth position and D-Orn to the second position. The same authors indicated that domains 3 and 4 may have a leucine-like binding activity, particularly domain 3, suggesting the presence of this amino acid at the corresponding position of the peptide. Due to this strong homology and the lack of a racemase region, we assume that the L-Ile present in the amino acid composition reported by Vanittanakom & Loeffler (1986) is present at the corresponding position 3. The remaining amino acids were putatively assigned on the basis of the following criteria: (i) homology of fengycin structural domains among themselves; and (ii) homology between the structural domains and known amino-acid-binding domains of other peptides, such as gramicidin S and surfactin. Following this approach, we assigned the third L-Glx to position 8 based on the high level of homology (87 and 93% identity) with the sequences surrounding the conserved PKG and GELC region of the first domain of PpsA and PpsC, respectively. The first domain of PpsD, corresponding to the seventh domain in the entire peptide, shows 54% identity with the first domain of GrsB (gramicidin

Fig. 2. Functional organization of the peptide synthetase complex encoded by the entire *pps* operon. The domains and the relative amino acids are indicated.

synthetase II) responsible for the binding of proline, thus allowing the assignment of this amino acid to the corresponding position of the peptide. In the sixth and ninth domains, a 500 aa portion of the carboxy-termini is approximately 50% homologous to protein portions with racemase activity of SrfA (surfactin synthetase I), GrsA (gramicidin synthetase I) and TycA (tyrocidin synthetase I). This would indicate the presence of D-amino acids at the corresponding positions. The only available position for the remaining L-Tyr could be the tenth, corresponding to the unique domain of PpsE. The similarity between domains 10 and 6, and the presence of a racemase region at the 3'-end of the latter, would indicate that this position is occupied by D-Tyr. This is in agreement with the observation made by Cosmina *et al.* (1993) that racemic stereoisomers of the same amino acid are characterized by similar motifs, though it is in disagreement with the amino acid sequence of plipastatin (synonymous with fengycin) reported on the basis of structural studies by Nishikiori *et al.* (1986) in *B. cereus* strain BMG302-ff67. Domain 9 containing the racemase function, sharing 59% identity with the third domain, and resembling a leucine-like, apolar amino-acid-binding domain, could be the D-Ala- or D-Val-binding site. Finally, the remaining polar D-allo-Thr would be placed at position 4, completing the group of four D-amino acids described by Vanittanakom & Loeffler (1986). Therefore, the amino acid sequence we propose is the following: L-Glx, D-Orn, L-Ile, D-allo-Thr, L-Glx, D-Tyr, L-Pro, L-Glx, D-Ala (D-Val), L-Tyr. The tenth amino-acid-binding domain is also followed by a thioesterase active site region responsible for the release of the synthesized peptide from the enzyme complex, as already described for the L- α -aminoadipyl-L-cysteinyl-D-valine and surfactin synthetase systems (van Liempt *et al.*, 1993). To our knowledge, this is the first attempt to define the amino acid sequence of fengycin on the basis of the DNA sequence of its synthetase operon.

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