Cloning and DNA sequence analysis of a bacteriocin gene of *Serratia marcescens*

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Serratia marcescens N28b synthesized and secreted a bacteriocin, with a molecular mass of 45 kDa, which was capable of inhibiting the growth of *Escherichia coli*. The expression of this bacteriocin was negligible unless induced with mitomycin C. The genes encoding the bacteriocin were cloned in plasmid pBR328. *E. coli* harbouring recombinant plasmid pBA189 or pBA289 expressed the *Serratia marcescens* N28b bacteriocin. The nucleotide sequence of the *bss* gene (*Serratia marcescens* N28b bacteriocin structural gene) was determined. The predicted amino acid sequence of the carboxy-terminal part of the bacteriocin 28b had a high degree of similarity to the pore-forming domains of colicins A, E1, B, N, Ia and Ib.

Introduction

Most studies on protein excretion in Gram-negative bacteria have been carried out in *Escherichia coli*, although this bacterium appears to be rather restricted in its ability to secrete proteins into the extracellular medium. From these studies two models for protein export have emerged: (i) proteins are synthesized as precursors with a signal peptide essential for proper protein secretion (Oliver, 1985; Regué & Wu, 1988); (ii) other exported proteins are synthesized without a recognizable signal peptide and their release is accomplished by one or more helper proteins (e.g. α -haemolysin, colicins A, E1, etc.) (Pugsley, 1988; De Graaf & Oudega, 1986).

So far, the best known colicins are encoded by colicinogenic plasmids. Usually, an operon containing three genes is involved in colicin synthesis and release. These genes are: a structural gene, an immunity protein gene and a gene coding for a bacteriocin release protein.

The bacteriocin release protein is a membrane polypeptide which is essential for colicin release (De Graaf & Oudega, 1986). The transcription of colicin operons is normally repressed by the LexA protein. This protein regulates the expression of the genes involved in the 'SOS' response (Walker, 1987). Little is known about the mechanisms of bacteriocin release in enterobacteria other than *E. coli*.

Serratia marcescens has been shown to produce bacteriocins upon induction with ultraviolet light and these bacteriocins have been used in typing *S. marcescens* isolates (Traub, 1980). Foulds & Shemin (1969) and Foulds (1972) isolated and characterized bacteriocin L from *S. marcescens*. Nevertheless, little is known about the genetic organization and release mechanisms of *S. marcescens* bacteriocins. In this work, the cloning of the genes coding for the *S. marcescens* N28b bacteriocin in pBR328 and the nucleotide sequence of the putative bacteriocin structural gene are reported.

Methods

Bacterial strains and media. *S. marcescens* N28b has been described previously (Gargallo-Viola, 1989). *E. coli* 5K F⁻ r_K⁻ m_K⁻ rpsL thr thi leu lacZ (Juárez *et al.*, 1984) was used as recipient for the genomic library of *S. marcescens*. *E. coli* XL1-Blue (Bullock *et al.*, 1987) was used in subcloning experiments with plasmid pSK (Bluescript II SK from Stratagene) as vector. All strains were grown on Trypticase Soy Broth (TSB) or Trypticase Soy Agar (TSA). Ampicillin (25 µg ml⁻¹) and

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Fig. 1. Restriction endonuclease map of the cloned bacteriocin DNA. Abbreviations: H, *Hind*III; S, *Sal*I; B, *Bgl*II. The production of bacteriocin by *E. coli* harbouring recombinant plasmids was determined by the agar overlay test (Pugsley & Oudega, 1987) using *E. coli* 5K(pBR328) as indicator strain.

Fig. 2. SP-Sephadex chromatography of void volume fractions from a QAE-Sephadex column. The column was eluted with 50 mM-phosphate buffer (pH 8.0) containing 0.1 M-NaCl. Bacteriocin peak fractions were pooled, dialysed and lyophilized. □, A_{280} ; ■, bacteriocin titre.

chloramphenicol ($25 \mu\text{g ml}^{-1}$) were added to the media when needed. For the induction of bacteriocin synthesis, mitomycin C (0.2 and $2.0 \mu\text{g ml}^{-1}$ for *E. coli* and *S. marcescens*, respectively) was added to cultures at a culture OD_{660} of 0.5 , and the cultures were grown for a further 2.5 h. The bacteriocin was harvested as previously described for bacteriocin L (Foulds & Shemin, 1969).

Bacteriocin assay. The production of bacteriocin on solid medium (TSA) was determined by the agar overlay test (Pugsley & Oudega, 1987). To quantify bacteriocin activity, twofold serial dilutions of bacteriocin preparations were placed on fresh TSA plates inoculated with $200 \mu\text{l}$ of an overnight culture of the indicator strain. The titre of bacteriocin activity was defined as the reciprocal of the largest dilution able to inhibit growth of *E. coli*.

Partial bacteriocin purification. Crude preparations of bacteriocin were subjected to QAE-Sephadex and SP-Sephadex chromatography. Fractions containing bacteriocinogenic activity were subjected to SDS-PAGE to determine the apparent molecular mass of the bacteriocin.

Construction of the genomic library. Isolation of total DNA, partial digestion with *Sau*3A and ligation to *Bam*HI-cut pBR328 DNA was

Fig. 3. (a) SDS-PAGE of a concentrated crude extracellular bacteriocin preparation (lane 2), the eluate from a polyacrylamide slice containing bacteriocinogenic activity (lane 3), bacteriocinogenic peak fractions from the SP-Sephadex column (lane 4) and molecular mass standards (lane 1: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.6 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa). (b) SDS-PAGE of [^{35}S]methionine-labelled products of an *in vitro* transcription/translation coupled system. Lane 2, *in vitro* products encoded by pBA.290 DNA; lane 1, *in vitro* products encoded by pSK DNA. The 31 kDa polypeptide corresponds to the TEM- β -lactamase encoded by pSK.

done as described by Maniatis *et al.* (1982). Standard methods were used to determine the restriction map of the recombinant plasmids and for the construction of subclones in plasmid pBR328 and pSK (Bluescript II SK from Stratagene).

15	ATAATCGCGGTGTC	915	AATCGGAAATTAAAGCCATCAAGGATGGCGTGCAGCCTGGCCGACAGGTATCAATAAGAT
75	ATCCGCGTTAAACGAGAATATCGGCATCTTAAACATAAACGAAATAGGATGAAAGCAT	280	N A E L K A I K D G V S L A A G I N K D
135	ATGCCGTGATTTAATTATGGTAAAGGAGATGGGACTAAGTGGAGTTCTGAACGCGGT	975	ATCCGGAAGAAAGATCGCGCGAATAACGCCAAACTCGCCAAGGATTTGGAGCGGGAATT
20	M P C F N Y G G K G D G T N W S S E R G	300	I A E K I G A K Y A K L A K D L E A G I
195	ACGGCCCTGAGCCGGGGGGCGGATAAAGCCATTCCGGCGATCGCGATCGCGCGCG	1035	CAAGGAAATATATCCGCAACGTCGAAGATCGGAGAAAACCTACGAGCAATTGACCAAG
40	T G P E P G G G D K G H S G D R D R G G	320	Q G K Y I R N V Q D A E K T Y E Q L T K
255	GCGGCGCTTGCAACAGCCCGAACAACAGACAGATAGCGGCGATCCAAAACGATCCGGCG	1095	GGCTGAACAAAAGCTCAAGCGCAGACAAAGCGCGATTGTCGCTGGCTGAAATG
60	A G V G N S P E Q Q I A A I Q N D P A	340	G L N K K L K A Q D K A A I V A W L K M
315	CTGCGCATGAAGCTGAGGCGGTGATTAAAGCGGCGGCGAGAAATTAAACCCGGATCGAAA	1155	ATCATGCGAAGCAGTATGCCCAATCGCGGGTATTGGCAAGGTCTTACCGGGTG
80	L R M K L E A V I K A A R R I N P D A K	360	I D A E Q Y A R N A R V L G K V F T G V
375	TTGCATATTGAAAGCTCAGCCCATCGGCGACGCTGAGCCTTCCGCCACCGGTTGACG	1215	GACTGGCGCATCAAGGGCGGATCTGTAAACGCCCGGATTGAAGGTTTCTCTACCGGC
100	L H I E S V S P S G T L S L S A T G L T	380	D W A I K G A D L V N A A I E G F S T G
435	GCAGATCAGCGAAGACATTTGCCCTGGGGGCTGTGTATGGGGTCAACCCCAAGGG	1275	AACTGGAAGCGTTCCGCAACCAAGCTCGAAGCGCTCGGTTTCGATCGGCGCAGGGTAT
120	A D Q A K H I G L G G L V M G V N A K G	400	N W K A F R N Q L E A L G L S I G A G Y
495	GTACAGTGGCATCGGGATATCGAAACCGGCATCGCGCAAGCCCTACCCCTGGC	1335	ACGTTTCGCGTATCGCGCGTTTTTCGCGCCGACGCTAGTGTCTGACGCGTGGCATT
140	V T V A I G D I E T G H A R K P S P P G	420	T L S A I A A F F A P T L V S T V G I
555	AAAGCGGCAACAAATGGCTGAATCCGGGCGAGATCGGCGCCTCTTCGTTAGGCAGTTT	1395	TTGCGCTTCGCTTACCTCTTTGCTGGCCACAGCTATATCGATCGGGAACGTGCGGG
160	K G G N N G L N A G Q I G A S S L G S F	440	F A F A Y L F G W A T S Y I D A E R A G
615	GTACAGACTCGCATCGGATCGGCGCTCAGCGGTCGGCATGGCAACGGCAAAACCGCG	1455	GAGTGGAGAAGTGGTTCGCTGACCTGTAACGCGCATGAGGGGCTGGCCCTCATTTTA
180	V T D S H R D R P V S G W H G N G K T G	449	E L E K W V A D L *
675	GAATTCTGACGACCGCACGACCGGAGTTACTACGGCTTTTCATCATTTTGAAGTGAA	1515	TCCCTCACACCCCTCTACCTCTTTTACACCAACCGGGCCACCGTCAGCAGCATTTGGCGA
735	E F S T T R T T G S Y Y G F H L K V E	1568	AGCGGCGCGTTCCGCGGAGGCGATCACCAAGCGGTCGATCCGATTTGACC
795	AAGCAAGACGAGTGGCACTACAGCCTCTACTATAGGCCAATAAGAACCGGCCGCG		
220	K Q D G L A T Y S L Y Y K A N K N R P A		
855	TTTATCGCGGTGAGAGGGGATAACCTGAATGCCATGGAGGTGAATATGCCAACGGC		
240	F I A V V R G D N L N A M E V K Y A N G		
885	AAGCGGTAAATACCCCGGCTCGGTAAACACGATCGTTAAGGAATTCGTCGRATATCAA		
260	K P V K S P G S V K T I V K E F V E Y Q		

Fig. 4. Nucleotide sequence of the *bss* gene. The predicted bacteriocin 28b amino acid sequence is shown below the DNA sequence. Restriction sites and the putative ribosome-binding site (S-D) are labelled above the DNA sequence. The putative transcriptional terminator is shown as inverted arrows.

Construction of unidirectional deletions. A 6.5 kbp *Hind*III-*Bgl*II DNA fragment from pBA289 insert was subcloned in the pSK vector. The resulting plasmid (pBA.SKHB) was digested with *Kpn*I and *Hind*III and a set of unidirectional deletions was constructed using an *Exo*III/Mung bean nuclease deletion kit (Stratagene). The shortest subclone still retaining bacteriocinogenic activity (pBA.191) was digested with *Sac*I and *Xba*I and another set of unidirectional deletions was constructed.

DNA-directed protein synthesis in vitro. *In vitro* transcription/translation was carried out by using [^{35} S]methionine from (Amersham) with the DNA-directed transcription/translation kit (Amersham), as instructed by the supplier. Radiolabelled proteins were resolved in SDS-PAGE (Laemmli, 1970) and identified by autoradiography.

DNA sequencing. Plasmid DNA obtained from minipreparations was denatured at 37 °C for 30 min in 200 mM-NaOH, 0.2 mM-EDTA, in a final volume of 20 μ l. The DNA was neutralized by the addition of sodium acetate (pH 5.5) to a final concentration of 0.3 M. The DNA was precipitated with ethanol, pelleted, washed and dried. DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) with a T7 DNA sequencing kit (Pharmacia) according to the supplier's instructions. 5'-[α - ^{35} S]Deoxyadenosine thiotriphosphate [> 1000 Ci mmol $^{-1}$ (> 37 TBq mmol $^{-1}$)] was obtained from NEN-Du Pont.

Amino acid sequence analysis. All alignments of the amino acid sequence derived from the nucleotide sequence were performed using the Multalin computer program (Corpet, 1988). The hydrophobic profile (window size: 7 amino acids) was calculated according to Eisenberg *et al.* (1984).

Results and Discussion

Bacteriocin production

S. marcescens N28b had bacteriocinogenic activity. This could be shown both on solid media (overlay test) and in broth cultures. The bacteriocinogenic activity was inducible by mitomycin C and could be detected in the cytoplasmic and extracellular fractions of exponentially growing cultures 2.5 h after induction. Without induction no bacteriocinogenic activity was detectable in exponentially growing broth cultures, although bacteriocinogenic activity could be detected in non-induced overnight cultures. So, the behaviour of this bacteriocin was similar to the *S. marcescens* bacteriocin L (Foulds & Shemin, 1969).

Localization of the bacteriocin genes

Since most colicins are plasmid encoded, the plasmid profile of *S. marcescens* N28b was studied. A 3 kb plasmid was detected in this strain. This plasmid turned out not to be related to bacteriocin production, since cured strains still remained bacteriocinogenic and *E. coli* 5K transformed with this plasmid did not produce bacteriocin (data not shown). Using different methods of plasmid preparation no other plasmids could be

Fig. 5. Hydrophobicity analysis (Eisenberg *et al.*, 1984) of the carboxy-terminal part of bacteriocin 28b (last 194 amino acid residues) (a) and of the pore-forming domain of colicin A (b).

detected, suggesting that the *S. marcescens* N28b bacteriocin could be chromosomally encoded.

Cloning of the bacteriocin genes

A genomic library of *S. marcescens* N28b DNA was constructed using plasmid pBR328 as a vector. *E. coli* 5K was transformed with the genomic library. From about 2000 clones screened two clones were found to produce bacteriocin. The two recombinant plasmids were purified and characterized. As shown in Fig. 1, both recombinant plasmids (pBA189 and pBA289) shared a region of DNA of about 9.5 kb. Several subclones were constructed from plasmid pBA289, either by removing restriction fragments or by unidirectional deletion with *Exonuclease* III and Mung bean nuclease. Each subclone was tested for bacteriocin production (Fig. 1). The results indicated that the genes encoding *S. marcescens* N28b bacteriocin are located in a region of about 2.0 kb.

Renaturation of bacteriocin after SDS-PAGE

Several proteins, including bacteriocin L, have been shown to be renaturable after SDS-PAGE (Lacks & Springhorn, 1980; Foulds, 1972). To test the behaviour of

Fig. 6. Alignment of the amino acid sequences of the amino-terminal part of bacteriocin 28b (first 255 amino acid residues) and colicin A (first 388 amino acid residues). The Multalin computer program (Corpet, 1988) was used. Identical amino acid residues are shown in bold type.

bacteriocin 28b, sonicated cell-free extracts of *E. coli* 5K(pBA189) and crude extracellular bacteriocin preparations from induced *S. marcescens* N28b cultures were subjected to SDS-PAGE; the gels were then sliced into 5 mm slices, protein was eluted in 50 mM-phosphate buffer (pH 7.0) and bacteriocinogenic activity was assayed. Bacteriocin 28b was renaturable, since protein from one eluted slice was still bacteriocinogenic (data not shown). When the protein from the eluted bacteriocinogenic slice was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250 protein bands were located in the 30 to 50 kDa region (see Fig. 3a, lane 3).

Partial purification of the bacteriocin

Crude extracellular bacteriocin preparations from *S. marcescens* N28b were applied to a QAE-Sephadex

column previously equilibrated with 50 mM-phosphate buffer (pH 8.0). Chromatographic separation was accomplished under starting conditions and bacteriocin activity was found in the void volume. The pooled void volume fractions from the QAE-Sephadex column were applied to an SP-Sephadex column previously equilibrated with 50 mM-phosphate buffer (pH 8.0). The column was washed sequentially with starting buffer, buffer containing 0.1 M-NaCl, and buffer containing 0.2 M-NaCl (pH 8.0). Bacteriocinogenic activity eluted as a single peak with phosphate buffer containing 0.1 M-NaCl (Fig. 2). The pooled fractions from the SP-Sephadex column were dialysed, lyophilized, and subjected to SDS-PAGE (Fig. 3a, lane 4). Two polypeptide bands were found, with molecular masses of 45 and 17 kDa. Since SDS-PAGE of eluted gel slices containing bacteriocinogenic activity showed polypeptide bands in

	260	270	280	290	300	310
28b	FVEYQNAELKAIKDGVSLAAGINKDIAEKIGAKYAKLAKDLEAGI...	QGKYIRNVQDAE				
A	VAEKAKDERELLEKTSELIAGMGDKIGEHLDKYKAIKDIADNIKNFQ	GKTIRSFDDAM				
B	KKEQENDEKTVLTKTSEVIIISVGDKVGEYLGDKYKALSREIAENINNF	QGKTIRSYDDAM				
N	KEEKEKNEKEALLKASELVSGMGDKLGEYLGKYNVAKVANDIKNFHGRNIRSYNEAM					
E1	KKAQNNLLNSQIKDAVDATVSFYQTLTEKYGEKYSKMAQELADKS...	KGKKIGNVNEAL				
Ia	EEKRKQDELKATKDAINFTEFLKSVSEKYGAKAEQLAREMAGQA...	KGKKIRNVVEAL				
Ib	EEKRKRDEINMVKDAIKLTSDFYRTIYDEFKGQASELAKELASVS...	QGQIKSVDDAL				
	320	330	340	350	360	370
28b	KTYEQLTKGLNKKLKAQDKAAIV.AWLKMDIAEQYARNARVLGKVFTGVDWA	IKGADLVN				
A	ASLNKITANPAMKINKADRDALVNAA.KHVDQAQDMANKLGNLSKAFKVADVVMKVEKVRE					
B	SSINKLMANPSLKINATDKEAIVNAA.KAFNAEDMGNKFAALGKTFKAADYA	IKANNIRE				
N	ASLNKVLANPKMKVNKSDKDAIVNAA.KQVNAKDMANKIGNLGKAFKVADL	AIKVEKIRE				
E1	AAFEKYKDVLNKKFSKADRDALFNA.LASVKYDDWAKHLDQFAKYLKITGHVSFGYDVVS					
Ia	KTYEKYRADINKKINAKDRAAIAAA.LESVKLSDISSNLNRFSGRLGYAGKFTSLADWIT					
Ib	NAFDKFRNNLNKKYNIQDRMAISKA.LEAINQVHMAENFKLFSKAFGFTGKVIERYDVAV					
	380	390	400	410	420	
28b	AAIEGFSTGNWKAFRNQLEALGLS.IGAGYTLISAIAAFFAPTTLVSSTVGF	IFAFAY...L				
A	KSIEGYETGNWGPLMLEVESWVLSGIASSVALGIFSATLGAYALSLGVP	AIAGIAGILL				
B	KSIEGYQTNWGPLMLEVESWVISGMAVAVALSFLSLTGLSALIAFGLSAT	VGVFVGVI				
N	KSIEGYNTGNWGPLLLEVESWIIGGVVAGVAISLFGAVLSFLPIS.GLAVT	ALVIGVIMT				
E1	DILKIKDTGDWKPLFLTLEKKAADAGVSYVVALLESLLAGT....	TLGIWGIAI...V				
Ia	EFGKAVRTENWRPLFVKTEETIIAGNAATALVALVESILTGS....	ALGIIGYGL...L				
Ib	ELQKAVKTDNWRPFFVKLESLSAAGRAASAVTAWAFSVMLGT....	PVGILGFAI...I				
	430	440	449			
28b	FGWATSYIDAERAGELEKWVADL					
A	AAVVGALIDDKFADALNNEIIRPAH					
B	AGAIGAFIDDKFVDELNHKIIK					
N	ISYLSSEFIDANRVSNINNISSVIR					
E1	TGILCSYIDKNKLNTINEVLGI					
Ia	MAVTGALIDESLVEKANKFWGI					
Ib	MAVSALVNDKFIEQVNKLIGI					

Fig. 7. Alignments of the amino acid sequences of the carboxy-terminal part of bacteriocin 28b (194 amino acid residues) and the pore-forming domains of colicins A, B, N, E1, Ia and Ib. The Multalin computer program (Corpet, 1988) was used. Bacteriocin 28b amino acid residues identical to one or more from the other colicins are shown in **bold** type.

the 30 to 50 kDa region, it was concluded that the 45 kDa band corresponds to bacteriocin 28b. Moreover, a [³⁵S]methionine-labelled polypeptide of molecular mass 45 kDa was detected in the *in vitro* products programmed by pBA.290 DNA (the smallest subclone from pBA189 still conferring bacteriocinogenic activity), but not in the *in vitro* products programmed by pSK DNA (Fig. 3b). Taken together, these results suggested that the 28b bacteriocin had been cloned in *E. coli*. Since the 17 kDa polypeptide appears in the partial purified bacteriocin, but not in the *in vitro* transcription/translation system, most probably this polypeptide is not related to the bacteriocin.

Nucleotide sequence of the *S. marcescens* bacteriocin structural gene (*bss*)

A set of recombinant plasmids was used to determine the nucleotide sequence of the *bss* gene. A sequence of

1568 bp was determined (Fig. 4). The size of the *bss* open reading frame was 1347 bp. A putative ribosome-binding site was found 9 bp upstream of the *bss* gene. No -10 or -35 sequences were found in the 75 bp sequenced upstream of the open reading frame. An inverted repeat sequence was found after the stop codon. This inverted repeat sequence could be the transcription terminator of the *bss* gene.

Analysis of the deduced bacteriocin amino acid sequence

The predicted product of *bss* gene is a 449-amino-acid polypeptide with a calculated molecular mass of 47.5 kDa, which is close to the 45 kDa value obtained from the partially purified bacteriocin. Inspection of the predicted amino acid sequence reveals a glycine- and proline-rich amino-terminal region. The carboxy-terminal region is rich in lysine residues and contains a stretch of 44 uncharged hydrophobic amino acid residues

flanked by acidic residues. This feature is also found in the carboxy-terminal part of the pore-forming colicins. According to hydrophobicity profiles, this stretch of sequence could be a membrane-spanning sequence (Pattus *et al.*, 1990). The hydrophobicity profile of the carboxy-terminal part of bacteriocin 28b shows a high degree of similarity to the corresponding domain of colicin A (Fig. 5). The amino-terminal amino acid sequence alignment of bacteriocin N28b and colicin A (Fig. 6) shows a high degree of similarity in the first 45 residues, with 35 identical residues. The carboxy-terminal amino acid sequence alignment of bacteriocin 28b (from amino acid 256 to 449) to the pore-forming domains of several colicins (Fig. 7) also shows a significant degree of similarity. The similarity is not very strong but is spread throughout the entire carboxy-terminal sequence: 60% of the bacteriocin 28b residues are identical to one or more from the other colicins, although the level of similarity to each colicin is only 23 to 30%. On the basis of sequence similarity of the carboxy-terminal amino acid sequence (pore-forming domain), colicins have been grouped in two families, the colicin E1 family (including colicins E1, Ia and Ib) and the colicin A family (including colicins A, B and N) (Pattus *et al.*, 1990). Bacteriocin 28b appears to be in an intermediate position between the colicin A and colicin E1 families. These results suggest that bacteriocin 28b has a pore-forming ability and that this could be the killing mechanism of this bacteriocin.

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