Stability and function of the signal peptide of the pCloDF13-derived bacteriocin release protein

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The pCloDF13-derived bacteriocin release protein (BRP) is synthesized as a prelipoprotein with a signal peptide which remains stable after processing. This signal peptide accumulates in the cytoplasmic membrane and is, together with the mature BRP, required for efficient release of cloacin DF13. We investigated the structural requirements for stability of the BRP signal peptide by constructing hybrid signal peptides consisting of parts of the BRP and Lpp signal peptides. Signal peptide stability was investigated by pulse-labelling and pulse-chase experiments. To study the functioning of the BRP signal peptide, the hybrid constructs were tested for their ability to promote BRP-mediated cloacin DF13-release and their ability to affect the viability of the host cells. The results obtained suggest that the N-terminal part of the BRP signal peptide together with the C-terminal alanine residue are important for stability. When expressed as a separate entity, all mutant signal peptides that contain a part of the BRP signal peptide are capable of affecting cell viability. The results indicated a possible correlation between stability of the BRP signal peptide and cloacin DF13-release.

Keywords: bacteriocin release protein, cloacin DF13, signal peptide stability, processing, *Escherichia coli*

INTRODUCTION

Bacteriocin release proteins (BRPs, also called lysis proteins or kil proteins) are involved in the semi-specific transfer of colicins and cloacin DF13 across the cell envelope of *Escherichia coli*. Expression of BRPs also results in a decline in culture turbidity (quasi-lysis or 'lysis'), inhibition of colony formation on agar plates (lethality) and leakage of periplasmic proteins by host cells (Pugsley, 1984; De Graaf & Oudega, 1986).

The pCloDF13-derived BRP is synthesized as a precursor lipoprotein of 49 amino acid residues. The preBRP is slowly processed by the lipoprotein-specific signal peptidase II (SPaseII), rendering a lipid-modified mature BRP which is located primarily in the outer membrane of

E. coli (Oudega *et al.*, 1984; Van der Wal *et al.*, 1992). By constructing hybrid BRP-derived proteins containing sorting sequences for proteins located in the cytoplasmic membrane, it was shown that the mature BRP itself contains structural information for its outer-membrane localization (Gennity *et al.*, 1992). The signal peptides of most BRPs are unusual in that they are not proteolytically degraded after processing of the preBRP (Cavard *et al.*, 1987; Pugsley & Cole, 1987; Luirink *et al.*, 1989). The stable signal peptides accumulate in the cytoplasmic membrane (Howard *et al.*, 1991; Van der Wal *et al.*, 1992).

The role of the pCloDF13-derived stable BRP signal peptide has been investigated by genetically exchanging the stable BRP signal peptide for the unstable Lpp signal peptide. The resulting hybrid BRP, correctly targeted by the Lpp signal peptide (Gennity *et al.*, 1992; Van der Wal *et al.*, 1992), did not function in the release of cloacin DF13, but expression did result in quasi-lysis, lethality and leakage of periplasmic proteins (Luirink *et al.*, 1991).

Abbreviations: BRP, bacteriocin release protein; SPaseII, signal peptidase II.

The BRP signal peptide expressed alone was not able to provoke the release of cloacin DF13, but did cause quasi-lysis and lethality (Van der Wal *et al.*, 1992).

In this study we investigated the structural requirements for BRP signal peptide stability and the possible correlation between signal peptide stability and release of cloacin DF13. Hybrid signal peptides consisting of parts of the unstable Lpp signal peptide and parts of the stable BRP signal peptide, as well as mutant BRP signal peptides, were analysed with respect to stability and ability to function in cloacin DF13-release, quasi-lysis and lethality.

METHODS

Bacterial strains, media and plasmids. *E. coli* MV1190 $\Delta(lac-proAB)$ *thi supE* $\Delta(srl-recA)$ 306::Tn10(*tet*^r)[F':*traD36 proAB lacI^a lacZ* Δ M15] (Messing, 1983) and *E. coli* CJ236 *dut-1 ung-1 thi-1 relA1*; pCJ105 (Cm^r) (Joyce & Grindley, 1984; Kunkel *et al.*, 1987) were used as host strains for the construction of mutant genes and DNA sequencing. The *sup⁻* strain *E. coli* FTP4170 $\Delta(tonB trpAE)$ *argE*(Am) *glyV55* was used for studying the expression of BRP derivatives (Luirink *et al.*, 1989).

YT medium (Miller, 1972) was used for culturing *E. coli* strains MV1190 and CJ236, and also for routine culturing of *E. coli* FTP4170. YT medium supplemented with 20 mM MgCl₂, to reduce BRP induced quasi-lysis, was used in cloacin DF13-release experiments (Luirink *et al.*, 1986). M9 minimal medium (Miller, 1972), supplemented with 20 mM MgCl₂ and 0.5% sodium lactate, was used for the labelling of cells with [³H]amino acids. Proteose peptone beef extract (Tokunaga & Wu, 1984) was used for the labelling of cells with [³H]palmitic acid. Ampicillin (100 µg ml⁻¹) and tetracycline (12.5 µg ml⁻¹) were added to the culture media when required. IPTG was used to induce the synthesis of BRP and BRP-related mutant proteins. Mitomycin C was used at 500 ng ml⁻¹ to induce cloacin DF13 synthesis.

pJL22 (*SphI*) encoding the wild-type BRP, pJL32-T₁ encoding a hybrid Lpp-BRP, pJL38 encoding the stable BRP signal peptide alone and pJL40 encoding the unstable Lpp signal peptide alone were described previously (Luirink *et al.*, 1991; Van der Wal *et al.*, 1992) and were used either for the construction of mutant BRP genes or as a control in lysis experiments. The expression vector pINIIIA1 (Masui *et al.*, 1984) was used for the expression of all mutant BRP constructs. pJL25, a pACYC184-derivative compatible with the constructed pINIIIA1-derivatives, encodes cloacin DF13 under control of the mitomycin C-inducible SOS promoter (Luirink *et al.*, 1988), and was used to study the specific cloacin release in complementation experiments with various BRP constructs.

Recombinant DNA techniques. Plasmid DNA and M13 double-stranded DNA were isolated as described by Birnboim & Doly (1979). Transformation of cells was carried out as described by Mandel & Higa (1970). Site-directed mutagenesis was carried out as described by Kunkel *et al.* (1987), using the Muta-gene M13 *in vitro* Mutagenesis Kit (BioRad). Mutants were identified using the dideoxy-termination method of Sanger *et al.* (1977) with the *Taq* Dye Primer Cycle Sequencing Kit and the 373A Automated DNA Sequencer of Applied Biosystems. To confirm that only desired mutations had taken place, the complete DNA sequence of subcloned mutated gene fragments was determined. All other basic recombinant DNA techniques were carried out using methods described by Sambrook *et al.* (1989).

Construction of mutant BRP genes. To enable the exchange of signal peptide coding regions, a *NarI* site was introduced in the middle of the BRP signal peptide gene. For this construction, an *XbaI*-*EcoRI* fragment of pJL22(*SphI*) encompassing the coding region of the complete preBRP, was cloned into M13mp19 using polylinker restriction sites. A *NarI* site in the coding region of the BRP signal peptide was created by *in vitro* mutagenesis using the oligonucleotide 5' CTA TTT TTC TTG GCG CCT TG 3' (altered nucleotides are underlined). As a consequence, two triplets encoding amino acid residues 10 and 11 were changed from TTT ATA (Phe-Ile) to GGC GCC (Gly-Ala), resulting in M13mp19cl21.

Likewise, a *NarI* site was introduced in the middle of the Lpp signal peptide gene. For this mutagenesis, a M13mp18 construct containing the pJL32-T₁-derived 1.2 kb *SaI* fragment encoding the hybrid Lpp-BRP was used. The oligonucleotide 5' CAG GAT TAC GCG GCC CAG 3' was used to introduce the *NarI* site, thereby changing the ninth triplet from GCC (Ala) to GCG (Ala). This construct was named M13mp18cl24.

For the construction of a mutant preBRP with a hybrid Lpp-BRP signal peptide, a 0.4 kb *NarI* fragment of M13mp19cl21 was ligated into the 5.8 kb *NarI* fragment of M13mp18cl24, resulting in M13mp18(21S24L) encoding the preBRP 10Lpp10BRP-BRP. The hybrid gene was cloned into pINIIIA1, resulting in pJL42 (Table 1).

For the construction of an amber mutant of pJL42 encoding only the hybrid Lpp-BRP signal peptide, a 0.4 kb *HindIII* fragment from pJL42 was ligated into M13mp18. The resulting construct was used for the introduction of an amber mutation at the first triplet of the gene encoding the mature BRP with the oligonucleotide 5' GTT TGC CTG CTA TGC GAC CAG 3'. The mutated signal peptide was cloned into pINIIIA1 using polylinker restriction sites. The resulting plasmid encoding the 10Lpp10BRP signal peptide was designated pJL45 (Table 1).

To construct a mutant preBRP with a hybrid BRP-Lpp signal peptide, a small (1.2 kb) *NarI* fragment of M13mp18cl24 was ligated into the M13mp19cl21-derived large (6.6 kb) *NarI* fragment which resulted in M13mp19(21L24S), encoding the hybrid 9BRP11LppAla-BRP precursor. This hybrid gene was cloned into pINIIIA1, resulting in pJL44 (Table 1).

For the construction of an amber mutant encoding only the hybrid BRP-Lpp signal peptide, M13mp19(21L24S) was used to introduce an amber mutation at the first triplet of the gene encoding the mature BRP with the oligonucleotide 5' CTG CTG GCA GGT TGC TAG GCA AAC TA 3' thereby reverting the last alanine residue of the signal peptide into a glycine residue. The mutated fragment encoding the hybrid 9BRP12Lpp signal peptide was cloned into pINIIIA1, resulting in pJL46 (Table 1).

For the construction of a mutant preBRP with a BRP signal peptide mutated in the hydrophobic core region, the complete hybrid BRP gene from M13mp19cl21, encoding 9BRPGlyAla10BRP-BRP was cloned into pINIIIA1, resulting in pJL47 (Table 1).

For the construction of an amber mutant encoding only the BRP signal peptide mutated in the hydrophobic core, a 0.4 kb *HindIII*-*EcoRI* fragment of M13mp19cl21 was cloned into M13mp18. An amber mutation was introduced at the first triplet of the gene encoding the mature BRP with the oligonucleotide 5' GTT TGC CTG CTA TGC GAC CAG 3'. The fragment was ligated into pINIIIA1, resulting in pJL49 encoding the mutated 9BRPGlyAla10BRP signal peptide (Table 1).

Unintentionally, the mutagenesis procedure used for the con-

Table 1. List of BRP derivatives used

The names of signal peptides used throughout the text are printed in *italics*, whereas the mature BRP is printed in **bold type**. The corresponding amino acid sequences are listed and aligned with respect to the SPaseII cleavage site. Lpp signal peptide-derived residues are underlined. For comparison, the complete amino acid sequence of the wild-type (*wt*) Lpp signal peptide is presented (Nakamura & Inouye, 1979). The primary sequence of mBRP is CQANYIRDVQGGTVAPSSSELGTGIAVQ (De Graaf & Oudega, 1986) and is referred to as CQA... in the table.

Plasmid	Gene product	Amino acid sequence
pJL22(<i>SphI</i>)	<i>wt</i> BRP- BRP	MKKAKAIFLFILIVSGFLLVA CQA...
pJL32-T ₁	<i>mut</i> Lpp- BRP	MKATKLVLGAVILGSTLLAA CQA...
pJL42	10Lpp10BRP- BRP	MKATKLVLGALIVSGFLLVA CQA...
pJL45	10Lpp10BRP	MKATKLVLGALIVSGFLLVA
pJL44	9BRP11LppAla- BRP	MKKAKAIFLGAVILGSTLLAA CQA...
pJL46	9BRP12Lpp	MKKAKAIFLGAVILGSTLLAG
pJL47	9BRPGlyAla10BRP- BRP	MKKAKAIFLGALIVSGFLLVA CQA...
pJL49	9BRPGlyAla10BRP	MKKAKAIFLGALIVSGFLLVA
pJL38	<i>wt</i> BRP	MKKAKAIFLFILIVSGFLLVA
pJL43	9BRPGly	MKKAKAIFLG
	<i>wt</i> Lpp	MKATKLVLGAVILGSTLLAG

struction of M13mp19cl21 also resulted in an amber mutation in the signal peptide coding region. This resulting plasmid encodes a peptide of 10 residues containing the nine N-terminal residues of the BRP signal peptide and, at the last position, a glycine residue. This peptide is referred to as 9BRPGly. A DNA fragment encoding this peptide was cloned into pINIIIA1, resulting in pJL43 (Table 1).

Analysis of gene products by discontinuous tricine SDS-PAGE. Gene products were analysed using highly cross-linked 16.5% (w/v) polyacrylamide in tricine SDS-PAGE, as described by Schägger & Von Jagow (1987).

Radiolabelling of proteins. For labelling experiments, exponentially growing cells (2×10^8 cells ml⁻¹) were induced with 1 mM IPTG. When required, globomycin, an inhibitor of SPaseII (Dev *et al.*, 1985), (a kind gift of M. Inukai, Sankyo Co., Tokyo, Japan) dissolved in dimethylsulfoxide (5 mg ml⁻¹) was added to a final concentration of 100 µg ml⁻¹, 5 min prior to induction with IPTG. After 10 min of induction, a mixture of [³H]amino acids (16 Ci mmol⁻¹; 0.59 TBq mmol⁻¹) or [³H]palmitic acid (53 Ci mmol⁻¹; 1.96 TBq mmol⁻¹) (Amersham) was added to a final concentration of 25 and 50 µCi ml⁻¹, respectively. Before labelling with [³H]amino acids, cells were collected by centrifugation and resuspended in 0.5 vols minimal medium. After 30 min of labelling, cells incubated with [³H]amino acids were collected by centrifugation, washed with PBS and dissolved in sample buffer. Labelling of fatty-acylated proteins with [³H]palmitic acid was terminated by adding trichloroacetic acid to a final concentration of 10% (w/v). For pulse-chase experiments, cells were labelled for 10 min with a mixture of [³H]amino acids (final concentration 20 µCi ml⁻¹) and chased with 0.2% Casamino acids, as described previously (Luirink *et al.*, 1989, 1991). Subsequently, the cells were collected by centrifugation, washed twice with methanol and dissolved in sample buffer. The samples were analysed by tricine SDS-PAGE and fluorography.

Determination of quasi-lysis, lethality and release of cloacin DF13, MDH and β -lactamase. Quasi-lysis was determined by measuring the optical density at 660 nm of induced cell cultures, as described previously (Luirink *et al.*, 1989). Lethality caused

by expression of BRP-derived constructs was determined as described previously (Luirink *et al.*, 1989). The amount of cloacin DF13 released into the culture medium or remaining in the cells was determined in triplicate with an ELISA as described previously (Luirink *et al.*, 1987). Double transformants of *E. coli* FTP4170 harbouring pJL25 and one of the various pINIIIA1-derivatives were employed. The double transformants were moderately induced for expression of BRP or BRP-derived constructs with 20 µM IPTG and fully induced for synthesis of cloacin DF13 with 500 ng mitomycin C ml⁻¹. The release of MDH and β -lactamase was measured as described by Bergmeyer & Bernt (1983) and Luirink *et al.* (1988).

RESULTS

Construction of mutant BRPs

In contrast to the Lpp signal peptide (Hussain *et al.*, 1982), the BRP signal peptide remains stable after processing of the preBRP (Luirink *et al.*, 1989). Furthermore, a BRP targeted by the unstable Lpp signal peptide does not function in the release of cloacin DF13 (Luirink *et al.*, 1991), which indicated that the accumulated stable signal peptide is involved in cloacin DF13-release. Therefore, the origin of signal peptide stability and functioning of the signal peptide with respect to cloacin DF13-release, quasi-lysis and lethality was further studied by constructing hybrid BRP-derived signal peptides.

Three different mutant signal peptides coupled to the mature BRP were constructed; a hybrid Lpp-BRP signal peptide, a hybrid BRP-Lpp signal peptide, and a BRP signal peptide mutated in the hydrophobic core region by substitution of two large hydrophobic residues for two small, less hydrophobic residues which are present at the same position in the Lpp signal peptide. The latter mutant was constructed in an attempt to destabilize the BRP signal peptide since it is known that this peptide contains more hydrophobic and bulky amino acid residues than the

Fig. 1 (a), (b), (e). For legend see facing page.

unstable Lpp signal peptide (Klein *et al.*, 1988). Furthermore, to study the effects of expression of the mutant BRP signal peptides alone, amber mutants were made by

introducing a stop codon at the first triplet of the mature BRP coding region. In addition, a truncated BRP signal peptide was created containing only nine amino acid

Fig. 1. Identification of plasmid-encoded gene products by tricine SDS-PAGE and fluorography. Cells of *E. coli* FTP4170 harbouring either pINIIIA1 or one of the various pJL plasmids (as indicated at the top) were labelled with [³H]amino acids (a) or [³H]palmitic acid (b) and analysed as described in the text. The absence or presence of globomycin during labelling is indicated above the figure by – or +, respectively. By pulse-chase labelling with [³H]amino acids, precursor processing and signal peptide stability were investigated (c, d, e). After various time periods of chase (indicated in min above the figure) samples were taken and analysed as described in the text. The position of marker polypeptides (kDa) is indicated. Symbols: □, lipid-modified preBRPs; ■, mBRP; ★, stable BRP signal peptide; ▷, hybrid 10Lpp10BRP signal peptide and possible proteolytic fragments; ►, hybrid 9BRP11LppAla signal peptide; ☆, hybrid 9BRPGlyAla10BRP signal peptide; →, mature Lpp.

residues of the wild-type BRP signal peptide plus a C-terminal glycine residue. All mutated genes were sub-cloned in the expression vector pINIIIA1 under control

of the IPTG-inducible tandem *lpp-lac* promoter/operator. The primary sequences of the encoded peptides are listed in Table 1.

Expression of BRP-derivatives

The expression of mutant BRP-derivatives was studied in *E. coli* FTP4170 by labelling with [^3H]amino acids (Fig. 1a). Polypeptide bands representing the stable wild-type BRP signal peptide, encoded by pJL22(*SphI*) and pJL38 (Fig. 1a, lanes 3 and 7), and the mature lipid-modified BRP (mBRP), encoded by pJL22(*SphI*) and pJL32-T₁ (Fig. 1a, lanes 3 and 5), have been identified previously (Luirink *et al.*, 1991). Cells harbouring pJL42, pJL44 or pJL47, encoding hybrid preBRPs, all expressed the mBRP (Fig. 1a, lanes 8, 11 and 14). The band representing the pJL42-encoded mBRP (Fig. 1a, lane 8) was much less intense than the pJL22(*SphI*)-encoded mBRP (Fig. 1a, lane 3) indicating that either its expression or its rate of processing is affected.

In addition to the mBRP expressed by cells harbouring pJL44 (*9BRP11LppAla-BRP*), a polypeptide band comigrating with the wild-type BRP signal peptide was detected (Fig. 1a, lane 11). This band probably represents the pJL44-encoded *9BRP11LppAla* signal peptide (predicted M_r 2117) which is apparently stable.

Analysis of cells harbouring pJL47 (*9BRPGlyAla10BRP-BRP*) showed the presence of a polypeptide band (Fig. 1a, lane 14) which migrated slightly faster than the wild-type BRP signal peptide. This band probably represents the pJL47-encoded mutant signal peptide (predicted M_r 2191) which is apparently less stable than the wild-type BRP signal peptide, as can be deduced from its lower intensity. Alternatively, it could be a proteolytic fragment of the mutant signal peptide.

In cells harbouring pJL42 (*10Lpp10BRP-BRP*), the hybrid signal peptide was not detected (Fig. 1a, lane 8; predicted M_r 2045) indicating that it is not stable. It must be noted, however, that the pJL42-encoded mutant BRP is expressed (or processed) to a lesser extent than the wild-type BRP (see above).

To study the expression of the mutant signal peptides alone (not coupled to the mBRP), similar labelling experiments were carried out with *E. coli* FTP4170 harbouring either pJL45, pJL46, or pJL49 (see Table 1). As described in Methods, these plasmids contain an amber mutation immediately after the signal peptide coding region, and expression in the suppressor-negative *E. coli* strain FTP4170 should result in the synthesis of the signal peptides only. In addition, the fortuitously obtained pJL43, encoding a peptide of 10 residues, was tested. Cells expressing the stable wild-type BRP signal peptide without the mBRP (pJL38; Van der Wal *et al.*, 1992) were used as a control.

In cells harbouring pJL45 (*10Lpp10BRP*; predicted M_r 2045) a weak polypeptide band of lower intensity, and migrating slightly faster than the wild-type BRP signal peptide, was detected (Fig. 1a, lane 10). This band probably represents the pJL45-encoded mutant signal peptide which is apparently less stable than the wild-type BRP signal peptide. Alternatively, it could be a proteolytic fragment of the mutant signal peptide.

The pJL46-encoded *9BRP12Lpp* signal peptide (Fig. 1a, lane 13; predicted M_r 2013) was not detected, in contrast to the comparable cleaved-off pJL44-encoded hybrid signal peptide, indicating that it is unstable (see Discussion).

Analysis of cells expressing the mutant pJL49-encoded *9BRPGlyAla10BRP* signal peptide (predicted M_r 2191) revealed the presence of a polypeptide (Fig. 1a, lane 16) of which the intensity was almost as high as that of the wild-type BRP signal peptide. The peptide comigrated with the signal peptide expressed by pJL47-harbouring cells. This band probably represents the pJL49-encoded mutant signal peptide itself, or its proteolytic breakdown product, as pointed out for the pJL47-encoded cleaved-off mutant signal peptide.

Finally, the expression of the pJL43-encoded truncated signal peptide (*9BRPGly*; predicted M_r 1107) was studied (Fig. 1a, lane 17). This peptide could not be detected, possibly because of its small size.

Lipid-modification and processing by SPaseII

By labelling cells with [^3H]amino acids and [^3H]palmitic acid in the absence and presence of globomycin, an inhibitor of the prolipoprotein signal peptidase SPaseII (Dev *et al.*, 1985), the lipid-modification and processing of all mutant preBRPs was investigated (Fig. 1a and b).

The mBRPs expressed by cells harbouring pJL22(*SphI*), pJL32-T₁, pJL42, pJL44 or pJL47 were detected after labelling with [^3H]palmitic acid (Fig. 1b, lanes 3, 5, 7, 9 and 11), indicating that these mBRPs are all lipid-modified. Upon treatment with globomycin, the lipid-modified preBRPs accumulated at the expense of the mBRPs (Fig. 1a, lanes 4, 6, 9, 12 and 15; Fig. 1b, lanes 4, 6, 8, 10 and 12), indicating that all preBRPs are processed by SPaseII which can only cleave lipid-modified precursors (Wu & Tokunaga, 1986). Again, the pJL42-encoded preBRP and mBRP appeared less intense, which is probably caused by the lower expression of this construct (see Fig. 1b, lane 7).

Signal peptide stability and rates of processing

The stability of the signal peptides and the rates of processing of the various preBRPs were investigated by pulse-chase labelling with [^3H]amino acids (Fig. 1c, d and e).

As can be seen from the increase in intensity of the mBRP encoded by pJL44, the rate of processing of *9BRP11LppAla-BRP* (Fig. 1c, lanes 9–12) is comparable to that of the wild-type preBRP (Fig. 1d, lanes 1–4). The intensity of the pJL44-encoded cleaved-off *9BRP11LppAla* signal peptide increased throughout the chase period comparable to the cleaved-off wild-type signal peptide, indicating that these signal peptides are of similar stability.

The pJL46-encoded *9BRP12Lpp* signal peptide expressed

Table 2. Effects of expression of BRP-derivatives on cloacin DF13-release, quasi-lysis and lethality

Plasmid	Percentage BRP-mediated cloacin release*	Quasi-lysis†	Lethality‡ (mM IPTG)
pJL22(<i>SphI</i>)	30	ND	ND
pJL32-T ₁	0	ND	ND
pJL42	20	ND	ND
pJL44	33	ND	ND
pJL47	23	ND	ND
pJL45	ND	++	0.0625
pJL46	ND	+	0.125
pJL49	ND	+	0.125
pJL43	ND	+	0.250
pJL38	ND	+++	0.125
pJL40	ND	—	> 4

ND, Not determined

* Double transformants of *E. coli* FTP4170, harbouring pJL25 encoding cloacin DF13 and one of the various plasmids encoding a preBRP, were induced for the synthesis of cloacin DF13 and BRP. After 5 h, samples were taken and the amount of cloacin DF13 released was determined using an ELISA as described previously (Luirink *et al.*, 1987).

† Quasi-lysis was determined for *E. coli* FTP4170 cells expressing the mutant BRP-derivatives, by measuring the optical density at 660 nm of induced cell cultures up to 5 h after induction.

‡ Lethality was determined as described previously (Luirink *et al.*, 1989) by spotting stationary-phase cultures on broth agar plates containing different concentrations of IPTG. The data given are the IPTG concentrations at which colony forming was inhibited.

alone, however, was not detected (Fig. 1c, lanes 13–16), indicating that it is unstable.

The rate of processing of the pJL47-encoded *9BRPGlyAla10BRP-BRP* (Fig. 1d, lanes 9–12) was comparable to that of the wild-type preBRP, as could be deduced from the increase of mBRP during the chase. The intensity of the pJL47-encoded cleaved-off mutant signal peptide, however, remained low throughout the chase period. This peptide migrated slightly faster than the wild-type BRP signal peptide, as observed above (see Fig. 1a, lane 14). These observations indicated that the *9BRPGlyAla10BRP* signal peptide (or its proteolytic fragment) is not as stable as the wild-type BRP signal peptide, but more stable than the Lpp signal peptide.

The comparable signal peptide expressed alone (pJL49) did not accumulate to the same extent as the wild-type BRP signal peptide expressed alone (Fig. 1d, lanes 13–16; Fig. 1e, lanes 1–4), indicating again that the *9BRPGlyAla10BRP* signal peptide is less stable than the wild-type BRP signal peptide.

The kinetics of the processing of the pJL42-encoded *10Lpp10BRP-BRP* (Fig. 1c, lanes 1–4) seem roughly similar to these of the wild-type preBRP encoded by

pJL22(*SphI*) (Fig. 1d, lanes 1–4). As observed in the pulse labelling with [³H]amino acids and [³H]palmitic acid (Fig. 1a, lane 8; Fig. 1b, lane 7), the extent to which the pJL42-encoded mBRP accumulated is relatively low, indicative of impaired expression. The cleaved-off *10Lpp10BRP* signal peptide was not detected, indicating that this hybrid signal peptide is not stable.

In cells harbouring pJL45 encoding the *10Lpp10BRP* signal peptide alone (predicted *M_r* 2045), a fast migrating peptide could be detected. This band, which decreased in intensity during the chase period (Fig. 1c, lanes 5–8), probably represents the unstable hybrid *10Lpp10BRP* signal peptide or its proteolytic fragment.

Release of cloacin DF13

To study the stability of the mutant BRP signal peptides and their functioning in the release of cloacin DF13, cells harbouring pJL42, pJL44 or pJL47 were transformed with pJL25, encoding cloacin DF13 under control of the mitomycin C-inducible SOS promoter. The double transformants were moderately induced for expression of the BRP derivatives, and fully induced for synthesis of the pJL25-encoded cloacin DF13. Under conditions similar to the conditions used in this study, *E. coli* cells expressing the mBRP, targeted by the stable wild-type BRP signal peptide, specifically released 30 % of the total amount of cloacin DF13 produced within 5 h after induction. Cells expressing the mBRP targeted by the unstable Lpp signal peptide did not release any cloacin DF13 (Luirink *et al.*, 1991; Table 2).

Cells expressing the pJL44-encoded mBRP, targeted by the completely stable hybrid *9BRP11LppAla* signal peptide, released 33 % of the bacteriocin, comparable to the specific release by cells expressing the pJL22(*SphI*)-encoded wild-type BRP (Table 2). This indicated that the 11 C-terminal amino acid residues of the BRP signal peptide, substituted here by residues from the Lpp signal peptide, are not directly involved in cloacin DF13-release.

Cells expressing the pJL47-encoded mBRP, targeted by the mutant *9BRPGlyAla10BRP* signal peptide of intermediary stability, released 23 % of the bacteriocin (Table 2). This indicated that substitution of the original residues of the BRP signal peptide (Phe⁻¹² and Ile⁻¹¹) by residues from the Lpp signal peptide (Gly⁻¹² and Ala⁻¹¹) affected functioning of the BRP signal peptide in cloacin DF13-release.

Expression of the pJL42-encoded *10Lpp10BRP-BRP* caused a net release of 20 % (Table 2). This result is difficult to evaluate in view of the relatively low expression of the pJL42-encoded mBRP (Fig. 1a, lane 8; Fig. 1b, lane 7).

Effects of mutant BRP-derived signal peptides on quasi-lysis and lethality

The constructs encoding the mutant BRP-derived signal peptides (pJL45, pJL46, pJL49 and pJL43) allow us to investigate the effects of their expression on quasi-lysis

and lethality in the absence of the mBRP, which itself has these effects (Luirink *et al.*, 1991).

To investigate the effects on growth of expression of mutant signal peptides, cells were cultured in the presence and absence of IPTG, and the culture turbidity was monitored. In parallel, the growth of control cells harbouring pJL38 (encoding wild-type BRP signal peptide) or pJL40 (encoding wild-type Lpp signal peptide) was followed. Upon induction of the various mutant BRP-derived signal peptides, the cells exhibited lysis patterns of which the extent was decreased and/or of which the onset was delayed as compared with cells harbouring pJL38 (Table 2). Induction of the pJL40-encoded unstable Lpp signal peptide, however did not affect cell growth at all (Table 2), indicating that all mutant BRP-derived signal peptides affected the growth of host cells.

To investigate the effects on cell viability, host cells expressing the mutant BRP-derived signal peptides were tested for their colony forming ability on broth agar plates as described in Methods. Control cells harbouring the expression vector pINIIIA1 or pJL40 (wild-type Lpp signal peptide) were not affected by any IPTG concentration used, whereas colony formation of cells harbouring pJL38 (wild-type BRP signal peptide) was sensitive to IPTG at a concentration of 125 μ M (Table 2). Cells expressing the mutant BRP-derivatives were sensitive to IPTG at levels between 63 μ M and 250 μ M (Table 2).

Apparently, expression of the mutant BRP-derived signal peptides is lethal to the host cells, but from these studies there is no clear correlation between the stability of a particular mutant BRP-derived signal peptide and its effect on the growth of host cells.

DISCUSSION

The aim of this study was to determine which part of the BRP signal peptide is responsible for stability, and to investigate a possible correlation between the stability of the BRP-targeting signal peptide and its functioning in cloacin DF13-release, quasi-lysis and lethality. Hybrid signal peptides were created, derived from the stable BRP signal peptide and the unstable Lpp signal peptide. In addition, a mutant BRP-derived signal peptide was constructed by substitution of two amino acid residues in the hydrophobic core region. The mutant signal peptides were expressed in the context of the preBRP or as a separate entity. Expression of the former constructs provoked the leakage of periplasmic proteins into the culture medium (data not shown), indicating correct targeting of the mBRP to the *E. coli* outer membrane (Van der Wal *et al.*, 1992).

The cleaved-off 9BRP11LppAla signal peptide seems to be stable, indicating that the nine N-terminal residues of the BRP signal peptide, and/or the alanine residue, are important for signal peptide stability. Unexpectedly, the 9BRP12Lpp signal peptide expressed alone, which had a C-terminal glycine residue, was not stable. Apparently,

the C-terminal alanine residue, present in the cleaved-off 9BRP11LppAla signal peptide, is also important for stability. The reason for the instability of the 9BRP12Lpp signal peptide as compared to the 9BRP11LppAla signal peptide is not clear. Previously, we showed that the wild-type BRP signal peptide expressed alone is completely stable (see Fig. 1e) and accumulates in the cytoplasmic membrane (Van der Wal *et al.*, 1992). An explanation might be that the 9BRP12Lpp signal peptide cannot properly insert into the cytoplasmic membrane and is degraded in the cytoplasm. Another explanation might be that the 9BRP12Lpp signal peptide inserts properly into the cytoplasmic membrane, but is sensitive to the signal peptide peptidases, in contrast to the 9BRP11LppAla signal peptide. The signal peptides derived from the colicin A, E2/E3 and cloacin DF13 BRPs having a C-terminal alanine residue are stable (Cavard *et al.*, 1987; Pugsley & Cole, 1987; Luirink *et al.*, 1989), whereas the colicin E1 BRP signal peptide with a C-terminal glycine residue is unstable (Cavard, 1991), indicating that a C-terminal alanine residue is important for signal peptide stability. However, substitution of the C-terminal alanine residue of the colicin A BRP signal peptide by a glycine residue had little or no effect on signal peptide stability (S. P. Howard, personal communication), indicating that a C-terminal alanine residue is not the only prerequisite for signal peptide stability. This conclusion is supported by the observation that the 10Lpp10BRP signal peptide (pJL42) and the mutant Lpp signal peptide (pJL32-T₁), both having a C-terminal alanine residue, are not stable.

The stable BRP signal peptide is extremely hydrophobic as compared to the unstable Lpp signal peptide and contains more bulky amino acid residues (Klein *et al.*, 1988). It has been suggested that these characteristics are related to the stability of the BRP signal peptide (Luirink *et al.*, 1991). To investigate this possibility, a mutant BRP signal peptide was constructed which contains two small less hydrophobic amino acid residues, including a residue known to destabilize α -helices. The resulting mutant 9BRPGlyAla10BRP signal peptide is presumed to have a different secondary structure and a decreased hydrophobicity. These changes appeared to destabilize the BRP signal peptide, albeit not completely. In this respect, it is interesting to note that the hydrophobic core region of the unstable colicin E1 BRP signal peptide is not as long as those of the stable signal peptides of the colicin A, E2/E3 and cloacin DF13 BRPs. In addition, the hydrophobic core region of the unstable colicin E1 BRP signal peptide contains a residue capable of destabilizing α -helices, in contrast to the stable BRP signal peptides (Klein *et al.*, 1988). Taken together, these results indicate that there is a correlation between signal peptide stability and either the length of its hydrophobic core region or its secondary structure.

There seems to be a discrepancy between the observations that the 9BRP11LppAla signal peptide is as stable as the wild-type BRP signal peptide, whereas the 9BRPGlyAla10BRP signal peptide has an intermediary stability. The latter mutant signal peptide only differs from the wild-type BRP signal peptide at positions -12

and -11, whereas the 9BRP11LppAla signal peptide contains, in addition, a stretch of nine residues derived from the Lpp signal peptide. Apparently, the additional Lpp signal peptide-derived residues compensate in an unknown way for the destabilizing effect of the glycine and alanine residues at positions -12 and -11. In addition, since the stable 9BRP11LppAla signal peptide contains a short hydrophobic core region, it appears that the length of this region is not the only factor determining stability.

When the mutant 10Lpp10BRP signal peptide was expressed in the context of the slowly processed preBRP, it was not detected. However, the mutant 10Lpp10BRP signal peptide could be detected when it was expressed as a separate entity, but it was rapidly degraded, indicating that this signal peptide is unstable. Apparently, the 11 N-terminal amino acid residues of the BRP signal peptide are important for signal peptide stability.

Taking these data together, it appears that both the 11 N-terminal amino acid residues and the C-terminal alanine residue of the BRP signal peptide together are important for its stability and that the length of the hydrophobic core region is not the only factor determining stability. However, the exact structural basis of signal peptide stability remains unknown.

When expressed as a separate entity, all BRP-derived signal peptides affected the growth and viability of the host cells, in contrast to the pJL40-encoded unstable Lpp signal peptide. The insertion of the more or less stable mutant signal peptides or their fragments into the cytoplasmic membrane might be responsible for the observed deleterious effects on growth and viability. Accumulation in the cytoplasmic membrane of the cleaved-off stable signal peptides of the colicin A- and cloacin DF13 BRPs, has been reported (Howard *et al.*, 1991; Van der Wal *et al.*, 1992). In addition, the latter signal peptide also accumulates in the cytoplasmic membrane when it is expressed as a separate entity (pJL38).

Previously obtained results suggested that the stable BRP signal peptide is involved in the release of cloacin DF13, since a BRP targeted by its own stable signal peptide mediated cloacin release, whereas a BRP targeted by the unstable Lpp signal peptide did not function in the release of cloacin DF13 (Luirink *et al.*, 1991). In the present study, three mutant signal peptides targeting the BRP were tested with respect to cloacin release. The pJL44-encoded stable mutant signal peptide was completely functional, whereas cloacin DF13-release by cells expressing BRPs targeted by the less stable (pJL42- and pJL47-encoded) mutant signal peptides was decreased. Although these observations indeed hint at a correlation between signal peptide stability and cloacin DF13-release, strong conclusions cannot be drawn, especially since expression of the pJL42-encoded BRP is relatively low, and secondly, the cleaved-off hybrid 10Lpp10BRP signal peptide was not detectable. We intend to generate a larger set of mutant signal peptides in a more random approach and test them for their capability to mediate cloacin DF13-release.

When stability of the BRP signal peptide is required for efficient release of cloacin DF13, the peptide might directly cooperate with the mature BRP in the formation of a protein pore more or less specific for cloacin DF13. Future studies will be directed towards the putative pore-forming abilities of the mature lipid-modified BRP and its stable signal peptide.

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