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Protein engineering of lantibiotics

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Abstract

Whereas protein engineering of enzymes and structural proteins nowadays is an established research tool for studying structure-function relationships of polypeptides and for improving their properties, the engineering of posttranslationally modified peptides, such as the lantibiotics, is just coming of age. The engineering of lantibiotics is less straightforward than that of unmodified proteins, since expression systems should be developed not only for the structural genes but also for the genes encoding the biosynthetic enzymes, immunity protein and regulatory proteins. Moreover, correct posttranslational modification of specific residues could in many cases be a prerequisite for production and secretion of the active lantibiotic, which limits the number of successful mutations one can apply. This paper describes the development of expression systems for the structural lantibiotic genes for nisin A, nisin Z, gallidermin, epidermin and Pep5, and gives examples of recently produced site-directed mutants of these lantibiotics. Characterization of the mutants yielded valuable information on biosynthetic requirements for production. Moreover, regions in the lantibiotics were identified that are of crucial importance for antimicrobial activity. Eventually, this knowledge will lead to the rational design of lantibiotics optimally suited for fighting specific undesirable microorganisms. The mutants are of additional value for studies directed towards the elucidation of the mode of action of lantibiotics.

Introduction

The cloning and sequencing of the first structural lantibiotic gene in 1988 (Schnell et al. 1988; Banerjee & Hansen 1988) and the very rapidly expanding number of other lantibiotic gene sequences, has stimulated intensive research towards the organization of gene clusters and elucidation of the biosynthetic pathways of lantibiotics. Moreover, it enabled a protein engineering approach as a powerful tool to identify lantibiotic structural elements important for antimicrobial activity and biosynthesis. In the First International Workshop on Lantibiotics in Bad Honnef in 1991, the development of expression systems for the nisin structural gene was reported and the preliminary characterization of the first mutants of nisin was described (Kuipers et al. 1991a). Three years later, at the Second International Workshop on Lantibiotics in Arnhem, an enormous progress in the field of protein engineering of the lantibiotics nisin A and Z, gallidermin, epidermin and Pep5 was demonstrated. The primary structures of these lantibiotics are depicted in Fig. 1. Two excellent reviews describe the structure, function, biosynthesis and mode of action of these

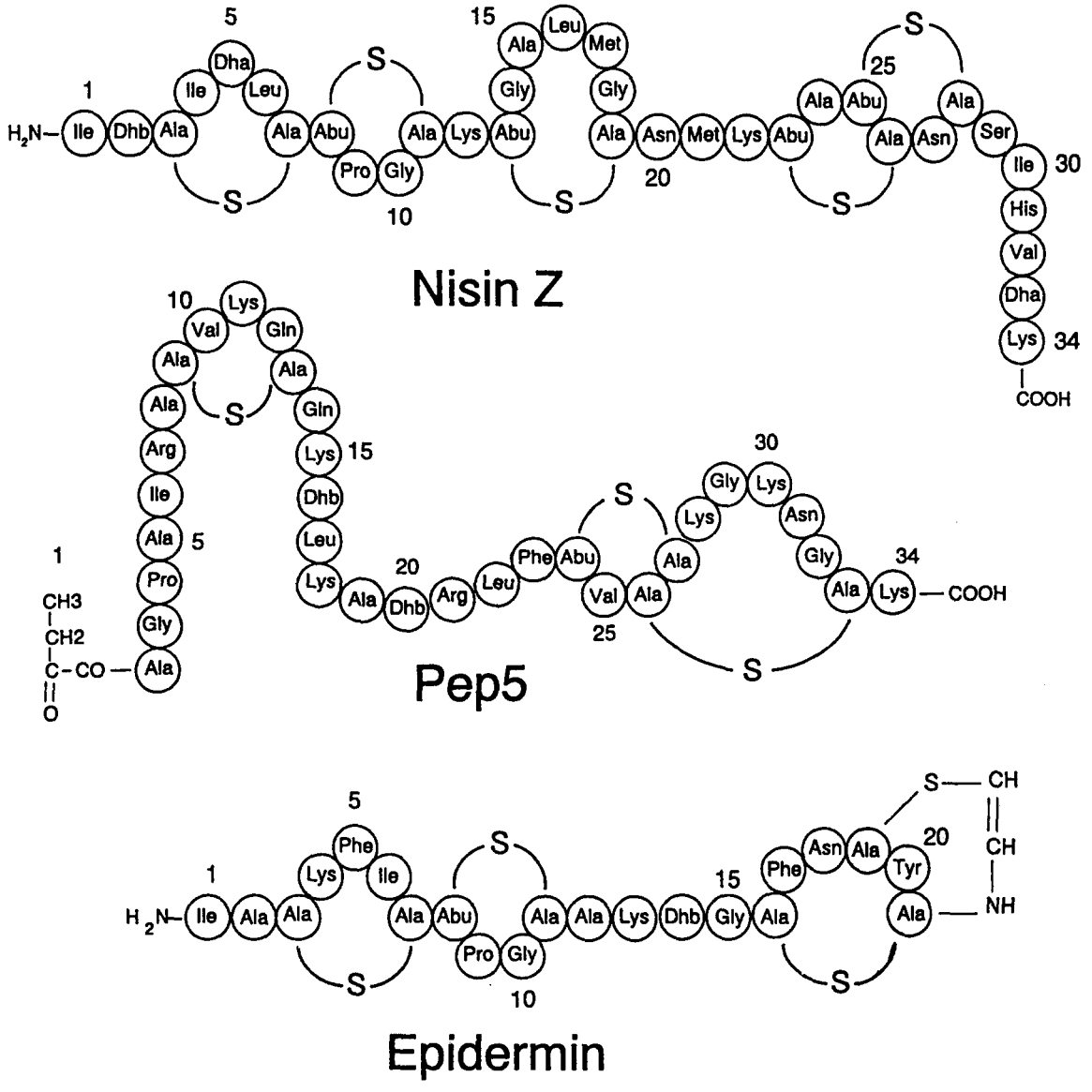

Fig. 1. Primary structures of nisin Z, Pep5 and epidermin. Nisin A is a natural variant of nisin Z containing a His residue at position 27. Gallidermin is a natural variant of epidermin containing a Leu residue at position 6.

and other lantibiotics (Jung, 1991; Sahl et al. 1995). In all afore-mentioned cases expression systems for the structural lantibiotic genes were developed, which will be briefly described in this overview, since the method chosen differs for each lantibiotic. Furthermore, the production, purification and structural and functional characterization of a great number of published and unpublished mutants of nisin, gallidermin, epidermin and Pep5 will be described, revealing common themes and differences between these lantibiotics. For instance, hinge regions appear to be present in each lantibiotic and seem to be of importance for antimicrobial activity, whereas introduction or removal of dehydrated residues can either stimulate or reduce activity. One of the great attractions of protein engineering of lantibiotics is the possibility of introducing or removing modified residues such as dehydroalanine, dehydrobutyrine and (β-methyl)lanthionine, to study their function in lantibiotic biosynthesis and action. In this respect, lantibiotic engineering offers a great opportunity to make use of the 'expanded' genetic code to generate more than the 19 other residues one can usually consider for a given residue. In fact, more than one mutant lantibiotic can sometimes be obtained from a

single point mutation, for instance because a certain residue will only be modified in a fraction of the peptide molecules. Eventually, these studies will open the way to the production of newly designed peptides with special properties, e.g. enhanced stability (lanthionine), redox-control (disulfides), presence of reactive groups (dehydroalanine), which might also find application outside the field of antimicrobial peptides. These and other examples will be discussed in the following sections.

Development of expression systems for lantibiotic structural genes

Lantibiotics are derived from precursor peptides encoded by structural genes and as such are amenable to protein engineering by changing the relevant DNA sequences. Hence, it is necessary to have the structural genes cloned on a suitable vector to enable mutagenesis to be undertaken and to allow the variant gene to be delivered back to a host where it will be expressed. In addition, processing of the engineered precursor requires the expression of all the other genes involved in biosynthesis of the mature lantibiotic. Expression systems have been described for nisin, subtilin, epidermin and Pep5, which employ different strategies for production of the engineered lantibiotic molecule. Two main approaches have been used involving either complementation of a deficiency in the structural gene of the host strain or substitution of the wild-type gene by a mutated copy using the technique of gene replacement.

Where plasmid complementation is to be employed it is desirable to first inactivate the structural gene encoding the prelantibiotic. This has been achieved in a nisin-producing *Lactococcus* strain by insertional inactivation of the *nisA* gene by the insertion sequence IS*905* (Dodd et al. 1992, 1994). The chromosomal deficiency is overcome by providing the missing function with a plasmid-encoded variant *nisA* gene. With this approach only the product of the variant *nisA* gene is subject to the processing specified by the other nisin biosynthetic proteins. Hence, this strategy has the advantage of ensuring the exclusive production of the variant prenisin and allows an instant assessment to be made of the activity of the resultant mutant peptide (Dodd et al. 1992). In a similar host strain, the disruption of *nisA* was achieved by replacing the wild-type *nisA* gene on the lactococcal chromosome by the truncated Δ*nisA* gene using replacement recombination (Kuipers et al. 1993a). The same method was employed to introduce site-directed mutant genes in the chromosome. Complementation of the hosts deficiency in the Δ*nisA* strain can also be achieved by plasmid-encoded (mutant) *nisA* or *nisZ* genes (Kuipers et al. 1993a).

An alternative approach to complementation has been successfully adopted by Kuipers et al. (1992) in which mutations are introduced into the plasmid-encoded structural gene of the closely related nisin Z (de Vos et al. 1993). The expression of these variant *nisZ* genes is achieved in a host strain producing nisin A, which can be separated from nisin Z and engineered variants by use of reversed-phase HPLC. In all these systems the structural gene is plasmid-encoded and its expression occurs independently of the other enzymes involved in nisin biosynthesis. The advantage of this system is that the sequences involved are more readily manipulated by a variety of molecular techniques. Cassette vectors have been developed in which the *nisA* gene is contained within small restriction fragments enabling mutations to be readily directed to specific parts of the nisin molecule (Dodd et al. 1995). In addition, alternative expression signals can be included in these plasmid constructs as demonstrated by the use of the lactococcal promoter *lacA* in the expression of *nisZ* variants (Kuipers et al. 1992) and *nisA* variants (Dodd et al. 1995). Furthermore, in the *nisZ* expression system the nisin yield has been shown to be increased 3-fold under the control of the strong *lacA* promoter (Kuipers et al. 1992).

Attempts to develop equivalent expression systems in other lantibiotic-producing bacteria have at first not proved successful when adopting a complementation approach (Liu and Hansen, 1992; Bierbaum et al. 1994a). Problems may arise when the structural gene is expressed on a multicopy plasmid, because overproduction of the mature lantibiotic could result in higher antimicrobial activity than the hosts immunity system can cope with. This was the case with *Bacillus subtilis* strains expressing the presubtilin gene, *spaS* (Liu and Hansen, 1992) and a *Staphylococcus epidermidis* strain expressing the precursor Pep5 gene, *pepA* (Bierbaum et al. 1994a). To overcome these problems expression systems have been developed in these strains in which the structural gene for the prelantibiotic was expressed at its natural genomic location. In the case of subtilin this involved removing the wild type *spaS* gene, in the chromosome of *Bacillus subtilis*, and replacing it with a mutant copy by gene replacement.

The Pep5 biosynthesis genes are plasmid encoded in *Staphylococcus epidermidis*. However, the construction of an expression system for engineering this lantibiotic proved difficult when employing a multicopy vector for the *pepA* gene, in combination with a low copynumber plasmid carrying the gene cluster with an inactivated *pepA* gene. To minimise this effect a low copy number vector was used for expression of the variant structural gene together with other Pep5 biosynthesis genes (Bierbaum et al. 1994a). The vector used, pGB9, harbored a 4.8 kb fragment of pED503, containing the Pep5 biosynthetic genes *pepPBC*, but not an intact *pepT*, in which mutant *pepI-pepA* fragments were inserted. As an expression host *Staphylococcus epidermidis* Pep5$^-$, a non Pep5-producing plasmid-free strain was employed. With this system several Pep5 mutants were produced (Bierbaum et al. 1994a, Bierbaum et al. 1994b).

An efficient expression system was also developed for epidermin and gallidermin structural genes (Ottenwälder et al. 1994). *Staphylococcus epidermidis* Tü*3298*/EMS6 (Augustin et al. 1992), an *epiA* mutant of the epidermin producer strain *Staphylococcus epidermidis* Tü*3298* showing an Epi$^-$ phenotype, was used to express natural and mutant gallidermin and epidermin species. The staphylococcal vector pT181mcs and the *E. coli- Staphylococcus* shuttle vector pCU1 (Augustin et al. 1992) were used as expression vectors for natural and mutant *gdmA* and *epiA* genes.

Moreover, gene replacement has been used to develop expression systems for engineered nisin in *Lactococcus lactis* (Kuipers et al. 1993a; Dodd et al. manuscript in preparation). Construction of a production strain involved the deletion of the entire *nisA* gene, together with the upstream nisin promoter as reported by Kuipers et al. (1993a). The resulting Nis$^-$ strain displayed immunity but only to significantly lower concentrations of nisin. In general, the production of an active nisin molecule can raise immunity to wild-type levels. Hence, the recovery of immunity could be used as a means of directly selecting strains that have acquired an intact *nisA* or variant *nisA* gene by gene replacement (Dodd et al. manuscript in preparation), if at least these mutants are able to induce transcription of the biosynthetic and immunity genes. The mechanism of induction and the problems related to the induction capacity of mutant nisins has been described recently (Kuipers et al., 1995). The advantages of a gene replacement approach are that normal gene-dosage and regulatory responses are maintained and the need to maintain plasmid-encoded functions is removed. It also has the advantage of retaining the balance between structural and biosynthetic genes. The future application of strains producing variant lantibiotics, particularly in the food industry, would also benefit from systems where the genetic modification has been kept to a minimum. With the gene replacement systems described for nisin (Kuipers et al. 1993a; Dodd et al. manuscript in preparation) and subtilin (Liu and Hansen, 1992) the only alterations to the genome are the specific mutations introduced in the structural gene.

Protein engineering of nisin

The nisin mutants and their main properties presented by Kuipers et al. in the Second Workshop as well as those reported previously by the same group (Kuipers et al. 1991a, 1992), are listed in Table 1. All mutants were made by site-directed mutagenesis (Kuipers et al. 1991b), and purified to homogeneity by RP-HPLC. For all mutants, except mutant I1W, the primary structure, including dehydrated residues and (β-methyl)lanthionines, was confirmed by 2D ^{1}H-NMR analysis.

The first engineered nisins reported were nisin Z species in which dehydrated residues (or their precursors Ser or Thr) were either introduced (M17Q/G18T and M17Q/G18Dhb) or replaced (Dha5Dhb) (Kuipers et al. 1991a, Kuipers et al. 1992). In all cases a 2- to 10-fold reduction in antimicrobial activity against three indicator strains was observed, except for M17Q/G18T nisin Z, which had twice the activity of wild-type nisin Z against *Micrococcus flavus*, but not against *Bacillus cereus* and *Streptococcus thermophilus*, indicating that a change in specificity occurred in this mutant. Mutant Dha5Dhb nisin Z was shown to possess a higher resistance against chemical degradation at low pH values than wild-type nisin Z (Rollema et al. 1995).

Recently, new nisin mutants were produced, which include I1W, T2S, S3T, K12P, T13C, M17W, N27K and H31K nisin Z, and N20P/M21P and ΔN20M21 nisin A (Kuipers et al. 1994). Introduction of the bulky Trp residue at position 1 reduces the activity of nisin, but not dramatically. This mutant will be useful for studying nisin-membrane interactions by fluorescence spectroscopy. Mutant T2S gave rise to a Dha residue at position 2 and interestingly this mutant nisin displayed a twofold higher antimicrobial activity against two target organisms, *e.g. Micrococcus flavus* and *Streptococcus thermophilus*. In contrast, the S3T mutation led

Table 1. Properties of engineered nisin species compared to properties of nisin A and nisin Z.

Mutation	Gene	Characteristic	Physical properties	Biological properties
I1W	*nisZ*	fluorescent label	not tested	similar activity
I1W/Thr-2	*nisZ*	unmodified Thr-2	not tested	reduced activity
T2S	*nisZ*	Dhb to Dha	not tested	increased activity
S3T	*nisZ*	Ala-S-Ala to Abu-S-Ala	not tested	very low activity
S5T	*nisZ*	Dha to Dhb	improved stability	reduced activity
K12P	*nisZ*	positive charge reduction	not tested	similar activity
T13C	*nisZ*	introduction of disulfide	reduction possible	reduced activity
M17Q/G18Dhb	*nisZ*	introduction novel Dhb	similar stability	similar activity
M17Q/G18T	*nisZ*	introduction unmodified Thr	similar stability	different spectrum
M17W	*nisZ*	fluorescent label	not tested	reduced activity
M17K	*nisZ*	lysine in ring 3	increased solubility	inactivated by trypsin
N20P/M21P	*nisA*	altered flexibility hinge region	not tested	reduced activity
ΔN20M21	*nisA*	partial removal hinge region	not tested	strongly reduced activity
N27K	*nisZ*	extra positive charge	improved solubility	similar activity
H31K	*nisZ*	altered positive charge	improved solubility	similar activity

increased activity:	> 120%
similar activity	80–100%
reduced activity	20–80%
strongly reduced activity	< 10%

Activities were measured by determination of MIC values of purified mutant nisins against *Micrococcus flavus*, as has been described previously (Kuipers et al, 1992).

to a β-methyllanthionine residue in ring 1, instead of the original lanthionine, and a dramatically decreased antimicrobial activity. Mutant K12P nisin Z showed similar antimicrobial properties as wild-type, indicating that a positive charge in the first half of nisin is not essential for nisin action, although it could play a role in enhancing the solubility of nisin. A mutant in which Thr-13 was replaced by Cys gave rise to a nisin species with a disulfide bond between residues 13 and 19, significantly lowering the antimicrobial activity. Upon reduction of this disulfide bond by DTT, almost complete loss of activity was observed. This indicates that the integrity of the third ring structure contributes to the antimicrobial activity, albeit that also the presence of two free sulfhydryl groups could have an adverse effect on activity. This notion was further supported by limited trypsinolysis of the slightly less active mutant M17K nisin Z. After opening of the third ring by specific cleavage behind Lys-17 the mutant lost almost all antimicrobial activity. Mutant M17W nisin Z was produced with the purpose of studying the binding affinity of nisin to lipid-water interfaces using Trp as a fluorescent probe. Preliminary results indicate that this nisin is less active than wild-type nisin, but that it can efficiently bind to micelles of *n*-dodecylphosphocholine (DPC) and to small unilamellar vesicles composed of different zwitterionic and negatively charged phospholipids (Gallay & Kuipers, unpublished results). The two mutations at positions 20 and 21 were made to restrict the mobility of the flexible hinge region (Van de Ven et al. 1991) between rings 3 and 4. Preliminary characterization showed that the two prolines indeed reduced the activity of nisin, while a deletion of the two residues lowered the antimicrobial activity more severely. A possible explanation for the latter phenomenon is that rings 4 and 5 are in another orientation relative to the first half of nisin, thereby destroying the amphipathic nature of the peptide. Two mutants were made with the objective of increasing solubility at neutral and higher pH, *i.e.* N27K and H31K. Both mutants indeed showed a 4- to 8-fold higher solubility at pH 7, which can be important when nisin is being applied at this pH in the presence of high salt concentrations (Rollema et al. 1995). Moreover, mutants were produced by random mutagenesis (Spee et al. 1993) and include L16P, M17V, M21L, T25A and I30T. These mutant nisins await further detailed characterization, but preliminary results indicate that mutants L16P,

Table 2. Activities in supernatants of mutant nisin A producing strains and immunity levels of producing strains

(mutant) nisin A	activity (% of wild-type)	immunity (μg/ml)
nisin A (MG1616)	100	>20
using complementing plasmids in strain FI7332		
H27Q	25	>20
H27Q, H32I	50	>20
H27Q, T23S	<1	>20
S5A	25	>20
S33A	10	>20
S5A, S33A	<1	>20
using gene replacement in FI7990		
S5A	100	>20
S33A	150	>20
S5A, S33A	100	>20
H27W	<1	2–5
S5A, H27W	10	>20
H27K	100	>20
H31K	25	>20
H27K, H31K	10	>20
K12L	10	10
I30W	<1	2–5

Activities were determined by agar-diffusion assays.

M17V, M21L and I30T are produced, secreted and active (Kuipers et al. unpublished results).

Mutations were also generated in the leader peptide to investigate the function of various residues (van der Meer et al. 1994). These mutations were made either in the most conserved region (consensus FNLDV at positions -18 to -14), or near the processing site (residues -4, -2 and -1) or in non-conserved residues. Mutations in the consensus sequence strongly affected either nisin biosynthesis or export, but mutations in non-conserved residues did not. Mutations near the processing site (residues -4 and -1, but not residue -2) did not affect nisin biosynthesis or export, but they inhibited processing by the leader peptidase NisP (van der Meer et al. 1993; Siezen et al. 1995), resulting in the secretion of a fully modified, inactive precursor nisin. A mutant in which the whole leader peptide of nisin was exchanged by the leader peptide of subtilin (a related lantibiotic from *Bacillus subtilis*), was also fully modified and secreted as a precursor nisin, indicating that the 43% of non-identical residues in the two leader peptides are not crucial for nisin biosynthesis (Kuipers et al. 1993b).

Another major contribution to the protein engineering of nisin was also presented during the Second Workshop (Horn et al. 1994). These authors used both complementation systems as well as gene replacement for the expression of *nisA* mutants. Interestingly, the levels of production of several of the mutants was quite different depending on the system used (Table 2). Generally, better results were obtained by gene replacement than by complementation. The reason for this phenomenon remains unclear, although it is likely that the uncoupling of transcription of the structural *nisA* gene from that of the downstream genes might have a negative effect on biosynthetic gene expression and hence the production level. It is also interesting to note that introduction of bulky Trp residues in the C-terminal part of the peptide (H27W and I30W) both severely reduce nisin activity. It remains to be seen if this is caused by a decrease in production level (hampered biosynthesis or secretion) or by a decrease in specific activity. Immunity levels of most mutant-producing strains were normal, although exceptions were found when gene replacement was used for expression. A good explanation for this phenomenon might be that the mutant nisins show a lower response in the NisRK mediated autoinduction pathway (Kuipers et al, 1995) of the transcription of the downstream genes, thus lowering expression of genes involved in providing immunity, such as *nisI* and *nisFEG* (see also chapter on lantibiotic biosynthesis of Entian & de Vos in this issue). Finally, it can be concluded that both Dha residues in nisin are not essential for activity, since replacement of Dha-5 and Dha-33 by Ala, still yields similar activity as produced by the wild-type strain (Table 2). As has been reported for subtilin the former mutation Dha-5-Ala eliminates nisin activity against outgrowing spores. This suggests that this activity against spores and the activity against vegetative cells involve distinct structural features of the nisin molecule.

Protein engineering of Pep5

By expressing the native *pepA* gene using the expression system mentioned earlier it became evident that two species of Pep5 were produced, one of which turned out to be fully matured Pep5 and the other one a Pep5 molecule that had a molecular mass that was 18 Da higher than that of Pep5. Further analysis revealed that this species had an additional serine residue and that probably ring A had not been formed. This species

had a considerably lower activity than wild-type Pep5 (Bierbaum et al. 1994b).

An interesting mutant in the hinge region of Pep5 was produced, i.e. K18P, which had a dramatically decreased activity against *Staphylococcus simulans* 22 (Bierbaum et al. 1994a). A change of the Phe residue at position 23, which is located just in front of the second ring of Pep5, into an Asp residue totally prevented the secretion of this mutant peptide, although intracellular accumulation of this peptide could be observed. Probably posttranslational modification reactions are hampered at a certain stage by the introduction of the Asp residue (Bierbaum et al. 1994a).

Two mutants in which the Dhb residues were replaced by Ala residues (Dhb16A and Dhb20A) showed considerably lower antimicrobial activities than wild-type Pep5 (Bierbaum et al. 1994b). The reason for this drastic decrease is currently under investigation. It seems possible that the mutations have an effect on the conformation or the flexibility of this middle region, or that the dehydrated residues play a direct role in the mechanism of antimicrobial action. Other mutants that were recently generated include the replacement of cysteine residues in the primary sequence to prevent ring formation. The mutant C33A was overproduced, although the main peptide produced consisted of only the amino acids 1–29, probably generated by proteolytic degradation behind position 29 (Bierbaum et al. 1994b). One can speculate that one of the functions of the third ring is to protect the peptide against this proteolytic degradation. The B ring of this peptide was shown to be present. This 1–29 mutant peptide had a lower antimicrobial activity than wild-type Pep5. The second cysteine mutant that was constructed, C27A Pep5, was produced as a mixture containing correctly and partially modified peptide as well as a degradation product (1–22 Pep5). The incorrectly modified peptide was devoid of ring C in addition to the expected lack of ring B, which indicates that formation of ring C is hampered in the absence of ring B. All these peptides displayed only a low antimicrobial activity. Another mutant was generated by replacement of the Ala-19 residue by a Cys. This residue could be involved in formation of a ring structure, which is currently under investigation (Bierbaum et al. 1994b).

Protein engineering of epidermin and gallidermin

Gallidermin and epidermin are highly homologous lantibiotics, which are ribosomally synthesized by *Staphylococcus gallinarum* Tü*3928* and *Staphylococcus epidermidis* Tü*3298*, respectively. The results obtained by a study of mutants generated by ethyl-methyl-sulfonate (EMS) treatment of the epidermin producer strain *S. epidermidis* Tü*3298* indicated that formation of ring A and B is important for completion of epidermin biosynthesis and epidermin production (Augustin et al. 1992). Two epidermin EMS-mutants, i.e. S3N and G10E could not be produced, probably because the mutations prevented correct formation of ring A and ring B, respectively. In order to analyze whether the intertwined rings C and D are also important for epidermin biosynthesis, production and activity, several amino acid alterations within the C-terminal part of the epidermin precursor peptide were introduced. Almost all C-terminal alterations of pre-epidermin, which affected thioether bridge formation at ring C or D, such as the deletion of the last two cysteine residues as well as the change of the Ser-19 residue into an Ala-19 residue, resulted in the complete loss of epidermin production (Table 3). These results strongly indicate that formation of all thioether amino acids is important for completion of epidermin biosynthesis and secretion.

The only C-terminal alteration of the precursor molecule which led to the formation of a secreted, antimicrobial active peptide was the S19T epidermin mutant. Ion-spray mass determination of this purified compound, revealed a molecular mass of 2178 Da which is in good agreement to the molecular mass expected for an epidermin derivative containing a Dhb-19 and an oxidative decarboxylated Cys-22 residue. Thioether bridging, i.e. formation of a C-terminal 2-aminovinyl-methylcysteine residue instead of the originally occurring 2-aminovinyl-cysteine residue, has still to be confirmed. However, epidermin S19T was only produced in trace amounts (0.4% of the production of gallidermin). This result may indicate that this mutant peptide is not efficiently recognized by the modification and/or secretion system.

A variety of gallidermin analogs have been engineered, which displayed quite interesting modulations of their antimicrobial activity against *M. luteus* and *A. cristallopoietes* (Table 3). With the exception of the T14S substitution, all of the newly introduced amino acid replacements showed complete modification. The T14S substitution in the gallidermin precursor peptide

Table 3. Mutant lantibiotics produced by *Staphylococcus epidermidis* Tü*3298*/EMS6 as host

Lantibiotic	molecular mass	antimicrobial activity (%)	
		M. luteus	*A. cristallopoites*
gallidermin			
wild-type	2164	100	100
T14S	2168	50	50
T14Dha	2150	100	100
T14A	2152	100	25
T14P	2178	3	6
L6V	2150	200	100
L6G	2108	50	13
epidermin			
S19A	not produced	-	-
S19T	2178 (trace amounts)	n.d.	n.d.
ΔC21/C22	not produced	-	-

n.d.: not determined.
Determination of antimicrobial activity was performed with a microdilution assay (Ottenwälder, 1994).

resulted in simultaneous production of a dehydrated (gallidermin Dhb14Dha, approx. 50%) and an unmodified derivative (gallidermin Dhb14S, approx. 50%). Incomplete dehydration has also been reported for a nisin mutant, in which the Thr at position 18 was not modified to dehydrobutyrine in 20% of the molecules (Kuipers et al. 1992) and Pep5 (Bierbaum et al. 1994a). The different gallidermin derivatives at position 14 displayed quite interesting differences with respect to their biological properties. Only gallidermin Dhb14Dha displayed a comparable antimicrobial activity to gallidermin against both indicator strains tested. Antimicrobial activity of the other derivatives at position 14, such as gallidermin Dhb14S, gallidermin Dhb14A and especially gallidermin Dhb14P was reduced (Table 3). This phenomenon might indicate that conformational changes in the middle region (Freund et al. 1991), such as an increase in peptide backbone flexibility or the introduction of a Pro-induced bend, are critical factors for antimicrobial activity. Since only gallidermin Dhb14Dha displayed comparable antimicrobial activities to gallidermin, this dehydroamino acid residue appears to be important for maintaining a special structure which is required for the pore forming activity. Interestingly, mutant Dhb14P appeared less sensitive to the action of trypsin, which shows that reducing susceptibility against proteases is feasible by protein engineering. Several other amino acid residues are also crucial for antimicrobial activity. The differences in activity of epidermin (Ile-6), gallidermin (Leu-6) and the L6V and L6G derivatives of gallidermin clearly indicate that the residue at this position plays an important role in the antimicrobial activity of gallidermin/epidermin as well. L6V gallidermin, for instance, was twice as active as gallidermin at least against *M. luteus*, whereas L6G gallidermin was less active (Table 3). Different susceptibilities of indicator strains might be caused by differences in membrane phospholipid composition, differences in cell wall composition or differences in membrane potentials.

Discussion and perspectives

More than 40 mutants of lantibiotics have already been generated and characterized. Although almost every mutation has resulted in a decreased antimicrobial activity and only a few (T2S nisin Z, M17Q/G18T nisin Z; L6V gallidermin) show increased activity against some target strains, these studies have provided a wealth of information on fundamental aspects of lantibiotic biosynthesis, secretion and processing. Mutations in non-standard residues have been produced, thereby expanding the possibilities of protein engineering. Mutant nisins with enhanced solubility (H27K and H31K nisin Z) have been obtained as well as more stable ones (Dha5Dhb nisin Z) (Rollema et al. 1995) and the proteolytically less sensitive Dhb14P mutant of gallidermin. Moreover, information on the importance of certain characteristics of specific residues for activity has been gained, such as the importance of the flexibility of the hinge region for activity, which seems to be a general phenomenon in type A lantibiotics. Engineering of residues involved in ring formation is risky, since most attempts to produce these species have failed sofar (e.g. S23A nisin A, S19A epidermin). What lacks at the moment is a detailed insight in the molecular interactions governing the action of lantibiotics. Especially the characterization of membranes of target organisms in relation to mutant-lantibiotic sensitivity is an urgent requirement for optimization of the action of the different lantibiotics. Another problem is related to the limited knowledge about the molecular mechanism underlying immunity: several mutant peptides might be lethal to the producing organism. Development of an *in vitro* modification system could provide a solution for this problem. Another alternative to circumvent the problem of reduced immunity to mutant lantibiotics would be to express the mutants with an

additional mutation at residue -4 or -1, in which case the mutant will be secreted as an inactive precursor protein that can be processed *in vitro* with a suitable protease, as has been shown for nisin Z precursors (van der Meer et al. 1993; Kuipers et al. 1993b).

Random mutagenesis also seems a powerful approach for generating lantibiotics with desired properties, provided that an adequate and sensitive phenotypic screening method is available. More futuristic goals to be reached are the development of newly designed peptides with posttranslational modifications for pharmaceutical use, the use of lantibiotics as vehicles for the secretion of other (non-lantibiotic) peptides, and combining functional domains of different lantibiotics into one peptide to change the antimicrobial spectrum.

References

Augustin J, Rosenstein R, Wieland B, Schneider U, Schnell N, Engelke G, Entian K.-D. & Götz F. (1992) Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of Staphylococcus epidermidis. Eur. J. Biochem. 204: 1149–1154

Banerjee S & Hansen JN (1988) Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J. Biol. Chem. 263: 9508–9514

Bierbaum G, Reis M, Szekat C & Sahl H-G (1994a) Construction of an expression system for engineering of the lantibiotic pep5. Appl. Environm. Microbiol. 60: 4332–4338

Bierbaum G, Reis M, Szekat C & Sahl H-G (1994b) Construction of an expression system for site directed mutagenesis in the pep5 biosynthesis operon. 2nd International Workshop on Lantibiotics, November 20–24 1994, Arnhem, the Netherlands

De Vos WM, Mulders JWM, Siezen RJ, Hugenholtz J & Kuipers OP (1993) Properties of nisin Z and the distribution of its gene, *nisZ*, in *Lactococcus lactis*. Appl. Environ. Microbiol. 59: 213–218

Dodd HM, Horn N, Hao Z & Gasson MJ (1992) A lactococcal expression system for engineered nisins. Appl. Environ. Microbiol. 58: 3683–3693

Dodd HM, Horn N & Gasson MJ (1994) Characterisation of IS905, a new multicopy insertion sequence identified in Lactococci. J. Bacteriol. 176: 3393–3396

Dodd HM, Horn N & Gasson MJ (1995) A cassette vector for protein engineering the lantibiotic nisin. Gene, submitted

Freund S, Jung G, Gutbrod O, Folkers G & Gibbons WA (1991) The three-dimensional solution structure of gallidermin determined by NMR-based molecular graphics, p. 91–102. In G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics, ESCOM Science Publishers, Leiden, Netherlands.

Horn N, Dodd HM & Gasson MJ (1994) Strategies for engineering nisin biosynthesis. 2nd International Workshop on Lantibiotics, November 20–24 1994, Arnhem, the Netherlands

Jung G (1991) Lantibiotics- ribosomally synthesized biologically active polypeptides containing sulfide bridges and α,β-didehydroamino acids. Angew. Chemie Int. Ed. Engl. 30: 1051–1068.

Kuipers OP, Yap WMGJ, Rollema HS, Beerthuyzen MM, Siezen RJ & de Vos WM (1991a) Expression of wild-type and mutant nisin genes in *Lactococcus lactis* in Nisin and Novel Lantibiotics, Sahl & Jung (Editors), Escom Publ, Leiden, 250–259

Kuipers OP, Boot HJ & de Vos WM (1991b) Improved site-directed mutagenesis method using PCR, Nucl. Acids. Res. 19: 4558

Kuipers OP, Rollema HS, Yap WMGJ, Boot HJ, Siezen RJ & de Vos, WM (1992) Engineering dehydrated amino acid residues in the antimicrobial peptide nisin. J. Biol. Chem. 267: 24340–24346

Kuipers OP, Beerthuyzen MM, Siezen RJ & de Vos WM (1993a) Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis* Requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. 216: 281–291

Kuipers OP, Rollema HS, de Vos WM & Siezen RJ (1993b) Biosynthesis and secretion of a precursor of nisin Z by *Lactococcus lactis*, directed by the leader peptide of the homologous lantibiotic subtilin from *Bacillus subtilis*. FEBS Letters 330: 23–27

Kuipers OP, Rollema HS, Bongers R, van den Bogaard P, Kosters H, de Vos WM & Siezen RJ (1994) Structure-function relationships of nisin studied by protein engineering. 2nd International Workshop on Lantibiotics, November 20–24 1994, Arnhem, the Netherlands

Kuipers OP, Beerthuyzen MM, de Ruyter PGGA, Luesink EJ & de Vos WM (1995) Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J. Biol. Chem. 270: 27299–27304

Liu W & Hansen JN (1992) Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. J. Biol. Chem. 267: 25078-25085

Ottenwälder B, Kupke T, Gnau V, Metzger J, Jung G & Götz F (1994) Isolation and characterization of genetically engineered gallidermin and epidermin analogues. 2nd International Workshop on Lantibiotics, November 20–24 1994, Arnhem, the Netherlands

Rollema HS, Kuipers OP, Both P, de Vos WM & Siezen RJ (1995) Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. Appl. Environ. Microbiol. 61: 2873–2878

Sahl H-G, Jack RW & Bierbaum G (1995) Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. Eur. J. Biochem. 230: 827–853

Schnell N, Entian K-D, Schneider U, Götz F, Zähner H, Kellner R & Jung G (1988) Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. Nature 333: 276-278

Siezen RJ, Rollema HS, Kuipers OP & de Vos WM (1995) Homology modeling of the *Lactococcus lactis* leader peptidase NisP and its interaction with the precursor of the lantibiotic nisin. Protein Engineering 8: 117–125

Spee J, de Vos WM & Kuipers OP (1993) Efficient random mutagenesis method with adjustable mutation frequency using PCR and dITP. Nucl. Acids. Res. 21: 777–778

Van der Meer JR, Polman J, Beerthuyzen MM, Siezen RJ, Kuipers OP & de Vos WM (1993) Characterization of the *Lactococcus lactis* nisin A operon genes *nisP* encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. 175: 2578–2588

Van der Meer JR, Rollema HS, Siezen RJ, Beerthuyzen MM, Kuipers OP & de Vos WM (1994) Influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by *Lactococcus lactis*. J. Biol. Chem. 269: 3555–3562

Van de Ven FJM, van den Hooven HW, Konings RNH & Hilbers CW (1991) NMR studies of lantibiotics. Eur. J. Biochem. 202: 1181–1188