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Cysteine *S*-glycosylation, a new post-translational modification found in glycopeptide bacteriocins

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ABSTRACT

_O_-glycosylation is a ubiquitous eukaryotic post-translational modification, whereas early reports of _S_-linked glycopeptides have never been verified. Prokaryotes also glycosylate proteins, but there are no confirmed examples of sidechain glycosylation in ribosomal antimicrobial polypeptides collectively known as bacteriocins. Here we show that glycocin F, a bacteriocin secreted by _Lactobacillus plantarum_ KW30, is modified by an _N_-acetylglucosamine β-_O_-linked to Ser18, and an _N_-acetylhexosamine _S_-linked to C-terminal Cys43. The _O_-linked _N_-acetylglucosamine is essential for bacteriostatic activity, and the C-terminus is required for full potency (IC_{50} 2 nM). Genomic context analysis identified diverse putative glycopeptide bacteriocins in Firmicutes. One of these, the reputed lantibiotic sublancin, was shown to contain a hexose _S_-linked to Cys22.

1. Introduction

Glycosylation is a ubiquitous eukaryotic post-translational modification, the glycans being typically linked to asparagine (*N*-linked) or serine/threonine (*O*-linked) sidechains and fulfilling physicochemical and/or molecular recognition roles [1]. There is also a growing appreciation of the extent and functional significance of bacterial glycoproteomes [2,3].

Prokaryotes secrete bacteriocins, ribosomally synthesised antimicrobial polypeptides that often have a narrow phylogenetic range of toxicity determined by specific interactions with receptor molecules and/or targets of inhibitory action [4]. Some bacteriocins exhibit unusual post-translational modifications [5,6], for example the C-terminal glycosyl ester linkage in microcin E492m [7], but there are no confirmed reports of bacteriocins with glycosylated sidechains.

Glycocin F (GccF, formerly plantaricin KW30), secreted by *Lactobacillus plantarum* KW30, is stable between pH 2-10, and at 100 °C for 2 h, and is unaffected by incubation with α-amylase and lysozyme [8]. These properties, deduced from studies on culture supernatant, are typical of bacteriocins from *L. plantarum* [9] and other lactic acid bacteria.

Here we present the molecular characterisation of purified GccF and show that it contains post-translational modifications not described previously in bacteriocins, including peptide cysteine *S*-glycosylation which is without verified biological precedent. Earlier accounts of naturally occurring cysteine (*S*-linked) glycopeptides [10,11] are almost forty-years old and in doubt because the peptide sequences (GenBank accessions P02728, P02729) have not been found in the human proteome.

Genomic context analysis indicated the sporadic occurrence of diverse putative *glyco*peptide bacterio*cins* (*glycocins*) in Firmicutes. We confirmed that one of these, the reputed lantibiotic sublancin 168 [12], is in fact an *S*-linked glycopeptide bacteriocin. These discoveries expand our awareness of the array of post-translational modifications available to confer antimicrobial and other properties on peptide scaffolds, and support the contention that bacteriocin diversity is greater than is currently recognised [13].

Abbreviations: GccF, glycocin F; ^{deOGlcNAc}GccF, *O*-deglycosylated GccF; $GccF_{1-41}$, peptide fragment 1–41 of GccF; GlcNAc, *N*-acetylglucosamine; HexNAc, *N*-acetylhexosamine; CD, circular dichroism; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; IC_{50}, concentration that decreases growth rate by 50%; ECD, electron capture dissociation; TFA, trifluoroacetic acid

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2. Materials and methods

2.1. Bacterial strains and culture conditions

L. plantarum strains (KW30, ATCC 8014) were grown at 30 °C in MRS medium (Merck). For GccF production KW30 was grown at 22 °C. For sublancin production *Bacillus subtilis* BR151 (BGSC, Ohio State University) was grown as described by Paik et al. [12].

2.2. Bacteriocin purification

Supernatant from a three-day 8 L KW30 culture was adjusted to pH 7 with NH_4OH and stirred overnight with 1 L phenyl Sepharose (FF, low substitution, GE Healthcare). The resin was packed into a glass column, washed with 3 L 2% NH_4HCO_3 and eluted with 2 L 2% NH_4HCO_3, 40% ethanol. Fractions (10 ml) containing active GccF were pooled, concentrated to ~12 ml by rotary evaporation, then purified by RP-HPLC (Jupiter 5 μm C_{18} 300 Å, 250 × 10 mm, Phenomenex; 1 ml injection, 5 ml min^{-1}, 25 min 0–50% B linear gradient. A: H_2O, 0.1% trifluoroacetic acid (TFA); B: acetonitrile, 0.08% TFA). GccF, eluting at ~40% B, was lyophilised (Fig. S1). The yield of purified GccF was 0.5–1 mg L^{-1} of culture. Active sublancin 168 was purified as described by Paik et al. [12].

2.3. Enzymatic dissection

GccF (1 mg ml^{-1} in 1 ml 1% NH_4HCO_3, pH 8.1) was incubated with 10 μg trypsin (Promega) overnight at room temperature. The digest was fractionated by RP-HPLC (Jupiter 5 μm C_{18} 300 Å, 250 × 4.6 mm, Phenomenex; 1 ml min^{-1}, gradient as in Section 2.2) to purify $GccF_{1-32}$ and $GccF_{33-43}$. A chymotryptic digest under similar conditions with a GccF:protease ratio of 50:1 by weight was incubated for 4.5 h and fractionated as above to purify peptide fragment 1–41 of GccF ($GccF_{1-41}$) and 'HX' (His42Cys43-*N*-acetylhexosamine (HexNAc)). GccF and $GccF_{1-32}$ (each 1 mg ml^{-1} in 1 ml 50 mM sodium acetate buffer, pH 4.5, 5 mM EDTA) were *O*-deglycosylated by adding 0.5 mg purified recombinant *N*-acetyl-β-D-glucosaminidase GcnA [14] and incubating overnight at room temperature. Reaction samples were analysed using MALDI-TOF MS (Micromass m@ldi) to detect the loss of *O*-linked HexNAc, and *O*-deglycosylated peptides purified as described for proteolytic fragments.

2.4. Analytical methods

GccF, reduced GccF, reduced and alkylated GccF, *O*-deglycosylated GccF (^{deOGlcNAc}GccF), $GccF_{1-41}$, $GccF_{1-32}$, $^{deOGlcNAc}GccF_{1-32}$, $GccF_{33-43}$, C-terminal peptide His42Cys43-HexNAc and sublancin were characterised by tandem mass spectrometry (PE SCIEX API 300 LC/MS/MS, PE Sciex Instruments; micrOTOF-Q, Bruker Daltonics; 9.4T APEX-Q Ultra Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), Bruker Daltonics). Circular dichroism (CD) spectra were collected on a Chirascan™ spectrometer (Applied Photophysics, U.K.). Experimental details of MS and CD spectroscopy, Edman and DNA sequencing, and bioinformatic methods are given in Supplementary data. Nucleotide sequence of the *gcc* cluster (6789 bp) was deposited in GenBank, accession number GU552553.

2.5. GccF activity assays

MRS medium containing 1% bacteriological agar was autoclaved then cooled to 40 °C. ATCC 8014 cells were added to ~3×10^6 cells ml^{-1} and 15 ml of this indicator agar was poured immediately into 7.2 cm diameter Petri dishes. Samples (2.5 μl) were pipetted onto indicator plates and incubated overnight at 30 °C. To detect antibacterial activity associated with peptides analysed by tricine SDS–PAGE, destained gels were overlaid with indicator agar. For IC_{50} determinations, the optical density (O.D. 600 nm) of 3 ml suspensions of ~3×10^7 cells ml^{-1} in MRS medium at ~30 °C was monitored (Hitachi U-1100 spectrophotometer) after addition of GccF and peptide fragments. IC_{50} is the concentration that halves the rate of O.D. 600 nm increase excluding the lag phase prior to inhibition. Tricine SDS–PAGE and LIVE/DEAD® cell assay methods are described in Supplementary data.

3. Results

3.1. Structural characterisation of GccF

The predicted monoisotopic mass of GccF is 4796.9197 Da, whereas the mass measured by FT-ICR-MS was 5199.0488 Da (shown as $[M+H]^+$ 5200.0561 Da in Fig. S2). GccF was analysed by Edman sequencing (Table S1) and FT-ICR-MS with electron capture dissociation (ECD) (Table S2), and shown to contain two types of post-translational modifications that account for the observed mass difference (Fig. 1A, $C_{226}H_{311}N_{57}O_{72}S_7$, theoretical monoisotopic mass 5199.0472 Da). Firstly, Edman sequencing identified two nested disulfide bonds (Cys5–Cys28 and Cys12–Cys21) – the same constrained $(C–X_6–C)_2$ 'hairpin architecture' was reported for sublancin 168 [12]. Secondly, FT-ICR-MS identified two HexNAcs, one linked to Ser18 and the other to Cys43. Partially glycosylated forms of GccF were not detected. Treatment with

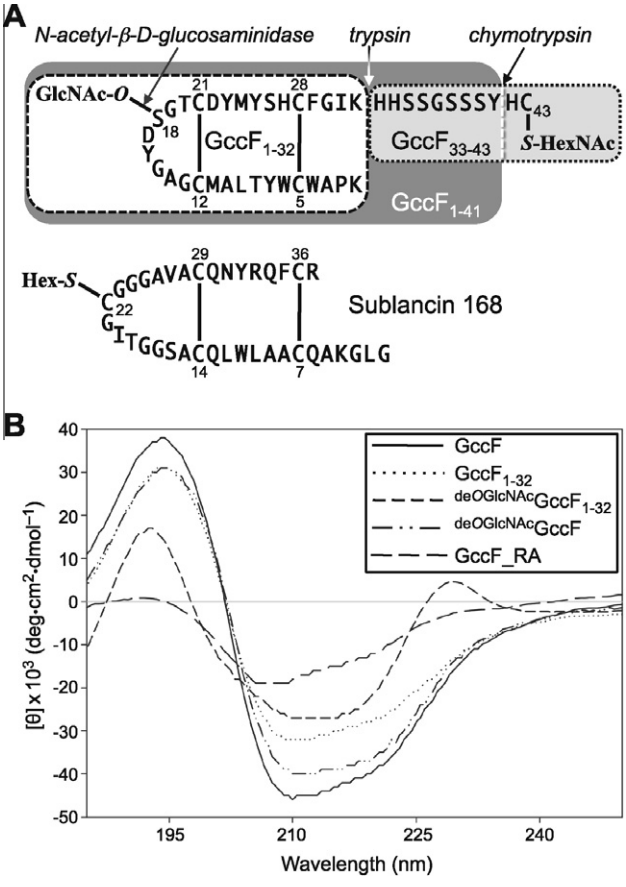

Fig. 1. (A) Schematic of GccF and sublancin 168, including enzymatic dissection of GccF. Trypsin cleavage produces $GccF_{1-32}$ and $GccF_{33-43}$, *N*-acetyl-β-D-glucosaminidase hydrolyses the *O*-glycosidic bond yielding ^{deOGlcNAc}GccF and $^{deOGlcNAc}GccF_{1-32}$, chymotrypsin cleavage yields His42Cys43-HexNAc, and partial chymotrypsin cleavage yields $GccF_{1-41}$. (B) CD spectra of GccF and fragments. GccF_RA, reduced and alkylated GccF.

the *N*-acetyl-β-D-glucosaminidase GcnA specifically released the Ser18-linked HexNAc from GccF and $GccF_{1-32}$ (Fig. S3), indicating that this HexNAc moiety is a β-*O*-linked *N*-acetylglucosamine (GlcNAc). Reduction of GccF CNBr peptides generated four free thiols that reacted with 4-vinyl pyridine (Table S1), whereas native (disulfide-bonded) GccF did not react with 4-vinyl pyridine or iodoacetamide. Edman sequencing, FT-ICR-MS and chemical modification results all supported the conclusion that GccF contains no free thiols; Cys5, Cys12, Cys21 and Cys28 thiols form disulfide bonds, and the C-terminal HexNAc is *S*-linked to the Cys43 thiol. Methyl esterification [15] of GccF provided evidence that the Cys43 HexNAc is not linked via the C-terminal carboxylate, as Asp17, Asp22 and the C-terminus were esterified.

CD spectra of GccF were dominated by bands at ~193 and ~210 nm, characteristic of helical structure, which diminished on reduction and alkylation (Fig. 1B). Compared to GccF, some of the decrease in 193 and 210 nm band intensity may be attributed to the loss of one (^{deOGlcNAc}GccF, $GccF_{1-32}$) or two ($^{deOGlcNAc}GccF_{1-32}$) HexNAcs [16]. However, the magnitude of the differences suggests that changes in secondary structure occurred when $GccF_{33-43}$ was removed, and that further changes occurred when $GccF_{1-32}$ was *O*-deglycosylated (a 230 nm band appeared), despite the disulfide bonds remaining intact throughout (Fig. S3).

3.2. GccF activity

Bacteriocins of lactic acid bacteria are typically cationic bactericidal peptides that kill by disrupting cell membrane integrity [4,17,18]. KW30 supernatant is apparently bactericidal to ATCC 8014 [8], and this differs from purified GccF (pI ~7.04) which had negligible bactericidal activity, *e.g.* incubation with 200 nM GccF for 5 h resulted in only 5% cell death (Fig. S4). Rather, purified GccF caused rapid and sustained bacteriostasis (IC_{50} ~2 nM; Fig. S5A).

GccF activity results are summarised in Fig. 2. ^{deOGlcNAc}GccF and $^{deOGlcNAc}GccF_{1-32}$ were inactive, showing that the *O*-linked GlcNAc is essential for activity. $GccF_{33-43}$ was also inactive, and did not increase the residual activity of $GccF_{1-32}$. Elevated IC_{50} values for C-terminal truncations $GccF_{1-41}$ (~130 nM) and $GccF_{1-32}$ (~350 nM) (Fig. S5B and C) showed that the C-terminus, and His42Cys43-HexNAc in particular, is required for full potency. Reduced GccF was inactive in indicator agar overlays of tricine SDS–PAGE gels (Fig. 2B, inset, lane 1).

Free GlcNAc protected cells against bacteriostasis by GccF and active fragments (Fig. 2A, compare columns 1 and 2). GlcNAc diffusing from samples in the left column decreased activity on the left side of the samples in column 2. Of twelve sugars tested (sorbitol, rhamnose, ManNAc, mannose, glucose, glucosamine, GalNAc, galactose, fructose, xylose, MurNAc, GlcNAc), only GlcNAc was protective (Fig. S6). In liquid culture the addition of 5 mM GlcNAc reversed bacteriostasis (Fig. 2B). During the ten-minute delay prior to resumption of growth there was a small but consistent decrease in O.D. 600 nm.

3.3. GccF gene and genomic context analysis

Bacteriocins may be chromosomally or plasmid encoded, and genes for regulation, maturation, export and immunity are typically adjacent and arranged in operons [4,17–19]. *GccF* is chromosomal [8] and encodes 64-amino acid preglycocin F including an N-terminal 21-amino acid double-glycine leader peptide (Fig. 3A). Northern blot analysis (Fig. S7) showed that *gccF* is transcribed from its own promoter, with an estimated 345 nt transcript size consistent with the *in silico* prediction of 357 nt starting at A6426 (Fig. 3A). The *gcc* gene cluster was predicted to contain eight genes (Fig. 3B, detailed analysis to be published elsewhere). There are no intergenic spaces between *gccA-E* encoding proteins with inferred functions consistent with GccF maturation, export and regulation (Table S3). GccA-D may form a membrane-bound complex in which: (1) glycosyltransferase GccA glycosylates Ser18 and/or Cys43 of preglycocin F, (2) the dedicated C39 (PF03412; cd02418, E-value: $2e^{-14}$)-ABC transporter GccB cleaves the leader peptide and exports GccF, and (3) exoplasmic membrane-anchored thioredoxin domain proteins GccC and/or GccD facilitate disulfide bond formation.

The 422-residue GccA contains a region (Val40-Arg267) with sequence similarity (E-value: $4e^{-29}$) to the β-1,4-glucosyltransferase domain cd02511 [20] found in enzymes involved in lipooligosaccharide biosynthesis, which are a subset of the CAZy database [21] family 2 glycosyltransferases. There is no significant sequence similarity between residues 268–422 and known proteins, including family 41 glycosyltransferases involved in the *O*-GlcNAc modification [22].

Applying the principles used to mine genomic data for bacteriocins [23], the co-location of glycosyltransferase and C39-ABC transporter genes was used as a criterion to identify *Bacillus–Lactobacillus–Streptococcus* group putative glycopeptide bacteriocin gene clusters. A subset of these is shown in Fig. 3B, and the corresponding bacteriocins in Fig. 3C.

ORFs encoding proteins with cd02511 domains are present in a few characterised bacteriocin gene clusters, notably those

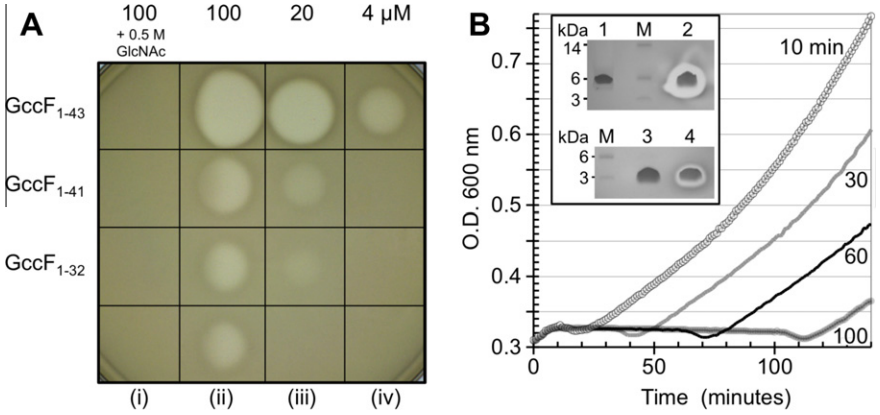

Fig. 2. Activity of GccF and fragment peptides. (A) Top three rows: 2.5 μL samples at peptide concentrations indicated were applied to an indicator plate. Bottom row: (i) 0.5 M GlcNAc; (ii) 100 μM $GccF_{1-32}$ + 200 μM $GccF_{33-43}$; (iii) 200 μM $GccF_{33-43}$; (iv) 200 μM ^{deOGlcNAc}GccF. (B) GlcNAc (5 mM) was added to ATCC 8014 cultures at 10, 30, 60 or 100 minutes after the addition of 8 nM GccF at 0 min. Inset: SDS–PAGE gels overlaid with indicator agar. (1) GccF treated with 120 mM dithiothreitol (20 °C, 3 h); M, marker; (2) non-reduced GccF; (3) $^{deOGlcNAc}GccF_{1-32}$ (inactive); (4), $GccF_{1-32}$ (attenuated activity).

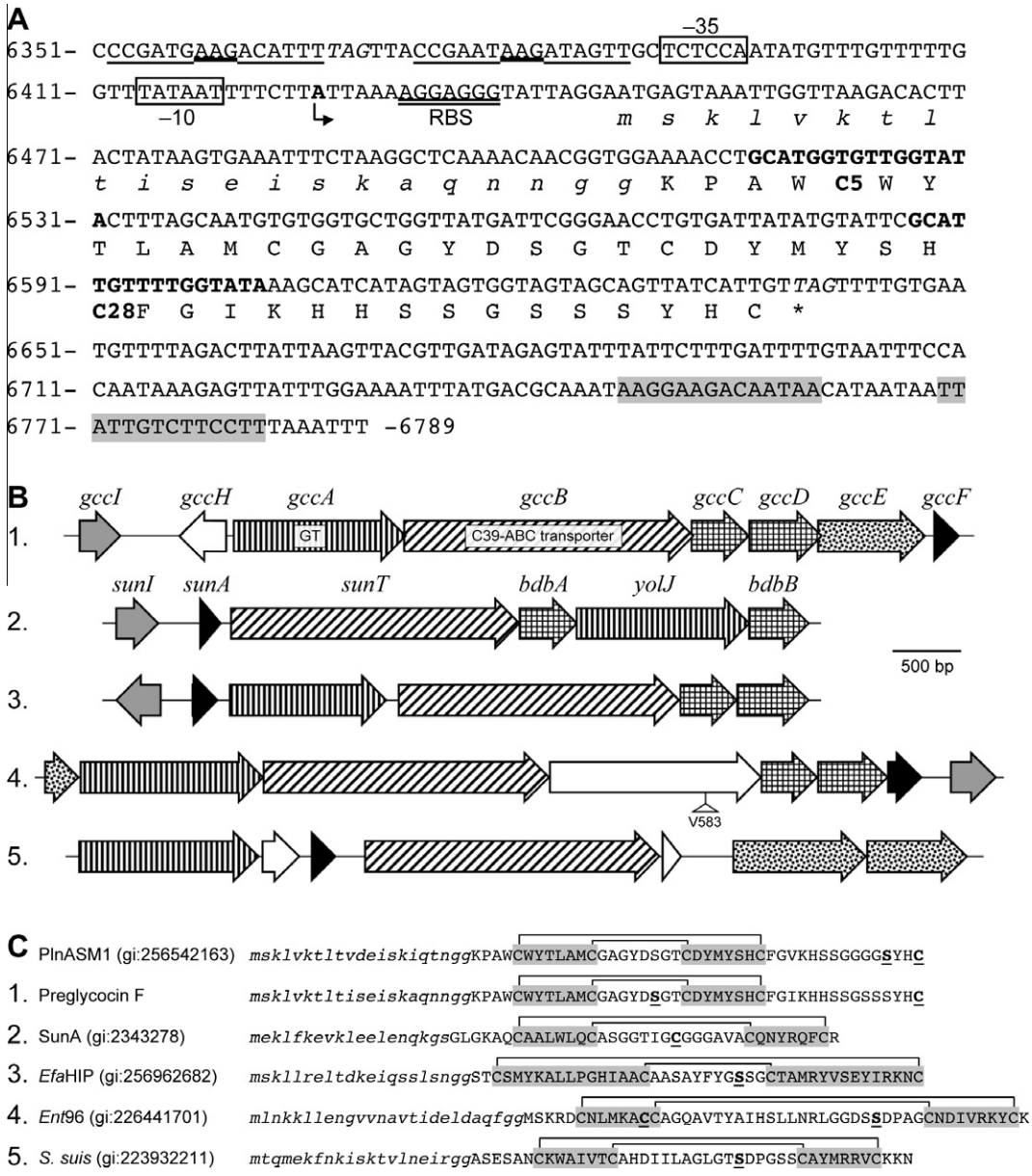

Fig. 3. The GccF gene, genomic context analysis, and putative glycopeptide bacteriocins. (A) DNA sequence of *gccF*. Numbering is from GenBank accession GU552553, and the translation to preglycocin F is shown. The *TAG* stop codon of the upstream *gccE* ORF lies within an imperfect repeat (underlined) with an 18 nt-spacing (AAG-N_{18}-AAG) characteristic of promoter elements recognised by LytTR response regulators that control transcription of some bacteriocin genes. The imperfect repeat encoding Cys5 and Cys28 is in bold type. A Rho-independent transcriptional terminator is highlighted ($\Delta G = -16.9$ kcal/mol). (B) Genomic context of *gccF* and representative putative glycopeptide bacteriocin genes. 1, *L. plantarum* KW30; 2, *B. subtilis* 168; 3, *Enterococcus faecalis* HIP11704; 4, *E. faecalis* TX0104; 5, *Streptococcus suis* 89-1591. Solid black arrows are *gccF* and putative bacteriocin genes (AA sequences of corresponding preglycocins are shown in (C)); vertical lines, glycosyltransferase (GT); diagonal lines, C39-ABC transporter; grid lines, thioredoxin domain protein; dotted pattern, putative signal transduction/regulation; solid grey, putative immunity; white, unknown. In 4, the triangle marks the position of a transposase in *E. faecalis* V583. DNA sequence for the enterocin 96 gene cluster of *E. faecalis* WHE 96 was not available; the *E. faecalis* TX0104 gene cluster encoding an identical prebacteriocin (gi:227517439) is shown here. (C) AA sequences of preglycocin F and putative preglycocins, including ASM1 [28], sublancin [12] and enterocin 96 [24]. Leader peptides (lowercase italics), and known (for GccF and SunA) and hypothetical disulfide bonds and glycosylation sites (bold, underlined) are indicated.

associated with the production of sublancin [12], PlnN [19] and enterocin 96 [24]. Although a role for peptide glycosyltransferases in the maturation of bacteriocins from Gram-positive bacteria has not previously been proposed, the molecular weights of sublancin and enterocin 96 are greater than predicted, suggesting that they could be glycopeptides.

3.4. Sublancin 168 is an S-linked glycopeptide bacteriocin

The initial report on sublancin 168 concluded that it contained lanthionine modifications [12], presumably introduced by Lan family enzymes. However, blastp searches (default parameters) using LanB, LanM and N-terminal regions of the recently described LanL proteins [25] as query sequences failed to detect Lan proteins in the *B. subtilis* 168 proteome. Given the proximity of *sunA* to *yolJ*, which encodes a cd02511 glycosyltransferase, the alternative hypothesis that sublancin is a glycopeptide was tested. The predicted monoisotopic mass of sublancin is 3717.7207 Da, whereas the mass measured by FT-ICR-MS was 3875.7454 Da, consistent with two disulfide bonds and a covalently attached hexose (Fig 1A; $C_{162}H_{254}N_{50}O_{51}S_5$, theoretical monoisotopic mass 3875.7422 Da). Subsequent ECD analysis (Table S2B) confirmed

that the hexose is linked to Cys22. After reduction of sublancin, alkylation with iodoacetamide led to a mass increase equal to the addition of four carbamidomethyl groups (Fig. S8B and C). However, the mass of native sublancin was unaffected by incubation with iodoacetimide (Fig. S8A and D), therefore sublancin, like GccF, has no free thiols and thus the hexose must be *S*-linked to Cys22.

4. Discusssion

Cysteine thiols are precursors for conformationally-constraining post-translational modifications such as disulfide bonds, cyclic thioethers and thiazole heterocycles found in a diverse ensemble of antimicrobial peptides including defensins, lantibiotics and thiopeptide antibiotics [5,6]. The sulfur to α-carbon bridges of subtilosin A [26] and thuricin CD [27] are further testament to the versatility of cysteine thiols as substrates for post-translational modification enzymes. Peptide *S*-glycosylation adds an extra dimension to cysteine post-translational modifications, and natural glycodiversification may prove to be an evolutionarily facile process for generating new and improved bacteriocins.

Recently Hata et al. [28] reported the characterisation of plantaricin ASM1, a homologue of GccF. Both bacteriocins are secreted by *L. plantarum* strains isolated from fermented corn products, one sourced from Japan (A-1) and the other from New Zealand (KW30). Although the post-translational modifications of ASM1 were not determined, residues 1-30 are identical to GccF (Fig. 3C), as is the difference between the theoretical and measured masses, suggesting that ASM1 also contains nested disulfides and two HexNAcs. In the Edman sequencing of both GccF and ASM1, a single serine gave no signal. For GccF this was the glycosylated Ser18, whereas for ASM1 it was reported to be Ser40, highlighting the potential for strain-specific glycodiversification.

The molecular receptor(s) and mechanism(s) of action of GccF remain to be elucidated, and may inform the design of new antibiotics. The inactivity of ^{deOGlcNAc}GccF and reversal of bacteriostasis by free GlcNAc suggest that the *O*-linked GlcNAc interacts reversibly with targets in susceptible cells. While the role of the *S*-linked HexNAc is enigmatic, it is interesting to consider the evolutionary and ecological consequences of 'baiting' bacteriocins with monosaccharides that are components of cell wall polymers and/or substrates for specific phosphotransferase system (PTS) transporters. The latter are numerous in *L. plantarum* [29], and transmembrane subunits of mannose PTS transporters are known receptors for class II bacteriocins [30]. Further, the *S*-glycosidic linkage could be relatively resistant to cleavage by extracellular and cell envelope glycosidases and transglycosylases, a strategy akin to incorporating D-amino acids into antimicrobial peptides to minimise proteolysis [31]. The monosaccharide moieties of GccF and sublancin may also protect against proteolysis, *e.g.* chymotrypsin cleavage at Tyr16 (Table S1). Proteolysis is an established mechanism of bacteriocin resistance and immunity [32].

Regarding sublancin, the interpretation of results presented by Paik et al. [12] could have been biased by the then reasonable hypothesis that this cysteine-rich modified bacteriocin was a lantibiotic, but that hypothesis is no longer tenable. According to Klaenhammer's classification scheme [17], both GccF and sublancin 168 are class IV bacteriocins, *i.e.* those modified with carbohydrate or lipid, and the 2005 proposal that class IV bacteriocins be withdrawn from classification schemes for lack of evidence [18] should therefore be revisited.

The simple genomic context criterion used here can identify only a fraction of glycopeptide bacteriocins. Given the ubiquity, diversity and shear number of potential peptide glycosyltransferase genes in prokaryotes, the phylogenetic distribution of glycopeptide bacteriocins may prove wider than other classes of modified bacteriocins. It will also be interesting to investigate whether peptide cysteine *S*-glycosylation is unique to bacteriocins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.023.

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