

The lumicins: novel bacteriocins from *Photorhabdus luminescens* with similarity to the uropathogenic-specific protein (USP) from uropathogenic *Escherichia coli*

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Received 27 June 2002; received in revised form 22 July 2002; accepted 23 July 2002

First published online 13 August 2002

Abstract

Bacteriocins are proteins produced by bacteria to destroy other bacteria occupying their ecological niche. *Photorhabdus luminescens* is an insect pathogenic bacterium carried by an entomopathogenic nematode and occupies several different niches in its life cycle. The nematode enters the insect and releases a single strain of *P. luminescens*. The bacteria then kill the host and the bacteria and nematodes replicate within the cadaver. Strikingly, at the end of the infection the cadaver is still occupied by a single strain of bacterium, suggesting that *P. luminescens* can destroy other bacteria entering, or present within, the insect. Here we describe four loci encoding 'lumicins' in *P. luminescens* subsp. *akhurstii* strain W14. The lumicins are novel bacteriocins capable of killing other strains of *Photorhabdus* and *Escherichia coli*. These loci predict killer proteins and multiple dual type immunity proteins with domains similar to pyocins and colicins. The killer proteins are chimeric in nature with multiple domains, one of which is similar to the uropathogenic-specific protein (USP) described from uropathogenic *E. coli*. The implications of these novel bacteriocins for the lifestyle of *Photorhabdus* and the potential role of USP as a bacteriocin in *E. coli* are discussed. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Bacteriocin; Colicin; Pyocin; Lumicin; Uropathogenic-specific protein; *Photorhabdus luminescens*

1. Introduction

Bacteriocins are proteins produced by bacteria that have antibiotic activity against closely related strains or species [1]. Bacteriocin-encoding loci contain genes encoding both a 'killer' protein, which has an enzymatic or permeabilizing activity on the target cell, and an 'immunity' protein [2] which provides the host cell with a defence against the toxic action of the killer protein. Different groups of these protein antibiotics have been named after the bacterial species in which they have been described. Thus the 'colicins', one of the most studied groups, derive from *Escherichia coli* [3] and the 'pyocins' are from *Pseudomonas*

aeruginosa (originally *Pseudomonas pyocyanea*) [1]. The colicins are plasmid-encoded, and their killer proteins show different modes of action including DNase/RNase activity or permeabilization of the bacterial cytoplasmic membrane [3]. Whilst within the host cell, the cytotoxic activity of the colicin killer protein is counteracted by specific immunity proteins that bind with high affinity to the toxic protein. Following release of the bacteriocin from the host cell, the colicins then enter the target cell via specific receptors on the outer membrane [4]. They are then translocated across the inner membrane via the Tol or Ton translocation system and the cleaved killer proteins then exert their toxic effect on the target cell [5]. The colicins are widespread in natural populations of *E. coli* and play an important part in the competitive ecology of *E. coli* strains [6,7]. A recent survey of the pyocins and related gene products suggests that domain swapping of

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different bacteriocins between different bacterial species has led to a diversity of bacteriocins in the γ -proteobacteria, including the insect pathogenic bacterium *Photobacterium* [1]. We are interested in determining the presence and likely function of bacteriocins in *Photobacterium*. We have previously detected putative bacteriocin-encoding loci in a partial gene cluster linked to the *toxin complex C* (*tcc*) locus of *Photobacterium luminescens* [8] and in a genomic sample sequence of *P. luminescens* subsp. *akhurstii* strain W14 [9].

Photobacterium and *Xenorhabdus* are Gram-negative entomopathogenic bacteria found in association with different species of entomopathogenic nematodes [10]. In each case, a specific strain of *Photobacterium* or *Xenorhabdus* bacteria is carried by a specific strain of nematode [11]. *P. luminescens* is carried within the gut of infective juvenile nematodes of the Heterorhabditidae and is released directly into the open blood circulatory system (hemocoel) of the insect host by the invading infective juvenile nematode [10]. Upon entering the hemocoel, the bacteria grow unhindered by the insect immune system and colonize specific tissues within the insect such as the midgut and fat body [12]. The insect then dies and both the bacteria and nematodes undergo subsequent rounds of replication within the cadaver [11]. Interestingly, at the end of the infection process, the insect is still only colonized by a single strain of *P. luminescens*, which can often be streaked directly onto an agar plate. This suggests not only that *Photobacterium* is able to overcome or suppress other bacteria introduced by different strains of nematode but also that it can repel bacteria invading from the soil. This ability of *Photobacterium* and *Xenorhabdus* to maintain a monoculture in the dead insect host suggests that they either secrete antibiotics and/or outcompete bacterial competitors. Indeed, both small molecule and protein antibiotics have been described previously. Several different small molecule antibiotics have been documented from *Photobacterium* and *Xenorhabdus* [13–20] and the presence of a phage tail-like bacteriocin, termed xenorhabdycin, has been detected in *Xenorhabdus nematophilus* [21].

To further describe the protein antibiotics produced, here we describe four new bacteriocin-encoding loci from *P. luminescens* subsp. *akhurstii* strain W14. These different loci, termed 'lumicins' or *lum* (after *luminescens*), predict killer proteins with both DNase and RNase activities. They also show unprecedented genomic organizations and killer protein fusions with partial similarity to the *E. coli* predicted uropathogenic-specific protein (USP). The *usp* gene has been described within a putative pathogenicity island from uropathogenic *E. coli* [22–24]. The role of the *usp* gene product in *E. coli*-mediated uropathogenesis itself remains unclear. However, it has most recently been proposed to be a bacteriocin enhancing the infectivity of *E. coli* in the urinary tract environment [25]. The lumicins reported here can kill other *Photobacterium* strains and also *E. coli*. These proteins may therefore

play an important part in maintaining a monoculture of a single *P. luminescens* strain within the insect cadaver or indeed within the gut of the nematode host itself. The broader association of predicted USP-like proteins with the insect pathogen *Photobacterium* suggests that they may be bacteriocins linked to pathogenicity islands in the Enterobacteriaceae, and supports the recently suggested hypothesis that *E. coli usp* encodes a bacteriocin [25].

2. Materials and methods

2.1. Bacterial strains, libraries and sequencing

The *Photobacterium* strains used in this study were: *P. luminescens* subsp. *akhurstii* strain W14, *P. luminescens* subsp. *temperata* strain K122, *P. luminescens* subsp. *laumondii* strain TTO1 and *Photobacterium asymbiotica* (a new species recently split from *P. luminescens* [26]) strain ATCC43949. The first three strains were isolated from their nematode symbionts from Florida, Ireland and Trinidad respectively. The third strain was isolated from a human wound, in the apparent absence of a nematode partner. Cosmid libraries with inserts averaging 30 kb were prepared from genomic DNA from each of these strains by MWG Biotec (Munich, Germany), as described previously [27]. Libraries were arrayed as individual *E. coli* clones in 96-well microtitre plates prior to activity or DNA based screening.

2.2. Activity and DNA based screening

Overlay halo assays for bacteriocin activity were performed on *Photobacterium* strains, cosmid libraries or plasmid subclones of DNA from the different strains used. Test cultures were stabbed into Luria Broth (LB) agar (or 2% PP3 agar plates for *Photobacterium*) containing appropriate antibiotics and incubated overnight at 37°C (or 28°C for *Photobacterium*). Plates were exposed to chloroform vapour for 15 min and then left for an hour to allow residual chloroform to evaporate. Plates were then overlaid with 5.0 ml molten 7 g l⁻¹ non-nutrient agar containing 50 μ l of the bacteriocin-sensitive strain (*E. coli* XL1 blue (pUC18), VSC257 or *Photobacterium*). Plates were incubated overnight at 37°C (28°C for *Photobacterium*) and inspected for a zone of clearance (inhibition of bacterial growth) or halo. In some cases plates were overlaid with 5.0 ml molten non-nutrient agar containing 50 microlitres of bacteriocin sensitive strain and test cultures were spotted (2.0 microlitres) onto the plates. After overnight incubation, plates were again inspected for halos. Tests were performed in the presence and absence of trypsin (1 mg ml⁻¹) in order to confirm that the antibiotic agent was likely to be a protein. Bacteriocin activity was attributed to specific open reading frames (ORFs) within the cosmids either by subcloning into pUC plasmid vectors or by inser-

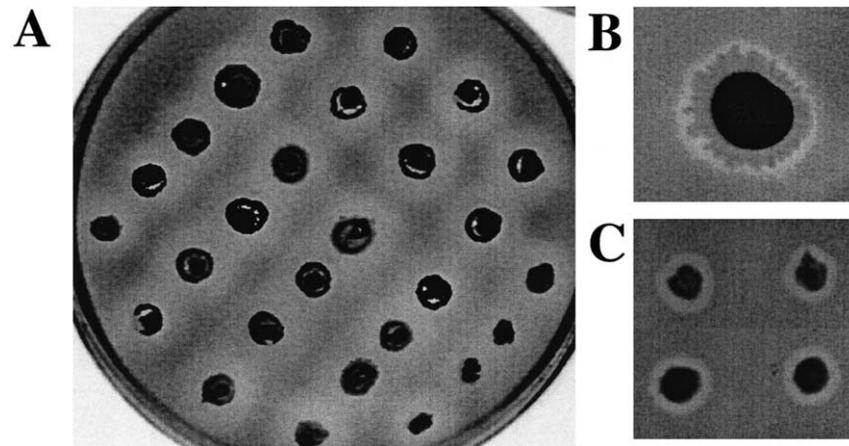


Fig. 1. Bacteriocin activity of different *Photorhabdus* strains and lumicin clones in *E. coli*. Clear zones of growth inhibition or halos can be seen surrounding bacteria with bacteriocin activity. A: *P. luminescens* strain W14 overlaid with *E. coli*. Note the completely clear zone of inhibition produced around a circular *P. luminescens* colony. B: *P. asymbiotica* overlaid with *E. coli*. Note the jagged interface between the *P. asymbiotica* colony and the zone of inhibition, which is characteristic of this strain and may indicate swarming of the bacterial colony. C: Zones of inhibition surrounding *lum1*, *lum2*, *lum3* and *lum4* (top left and clockwise) clones overlaid with *E. coli*.

tional mutagenesis of the corresponding cosmid with an $EZ::TN^{trp}<TET1>$ transposon (Epicentre).

To identify potential homologues of *lum1* we also used PCR based screening of DNA plate pools from the arrayed W14 cosmid library. PCR primers were designed from the partial sequence of the *lum1* locus previously described upstream from the *P. luminescens* W14 toxin complex *C* locus *tcc*. Primers used in the PCR were: forward, 5'-AAG CGGAGGTGGTAAACG AGA-3' and reverse, 5'-GCC CGC TAT CCC ACT ATC-3'.

2.3. Sequencing, phylogenies and accession numbers

Nucleotide sequences were determined on an ABI3700 capillary sequencer using the sequencing primers recommended within the $EZ::TN^{trp}<TET1>$ transposon (Epicentre). Phylogenies between the different killer protein ORFs, domains and immunity proteins were derived with Lasergene software (DNASar, Madison, WI, USA).

3. Results

3.1. Bacteriocin activity of strains and lumicin clones

Four different *Photorhabdus* strains, representing three different subspecies and one new species (*P. asymbiotica*), were tested for their ability to kill each other, and the unrelated *E. coli*, in overlay assays. All four *Photorhabdus* strains were able to kill each other, producing characteristic zones of clearing or halos in the overlaid bacteria. All four *Photorhabdus* strains were also active against *E. coli* and two examples are shown (Fig. 1A,B). The activity of lumicin containing cosmids and subclones from *P. luminescens* strain W14 was tested against *P. luminescens* K122 (data not shown) and *E. coli* (Table 1). Subclones containing the complete *lum1*, *lum2*, *lum3* or *lum4* locus all killed both *P. luminescens* strain K122 and *E. coli* (Fig. 1C). In all cases, killing activity was eliminated in the presence of trypsin, suggesting that the antibiotic is proteinaceous (data not shown).

Table 1
Photorhabdus strains and inhibitory activities of lumicin expressing clones

Strain or locus	Species or clone	Source
<i>Photorhabdus</i> species and subspecies		
W14	<i>P. luminescens</i> subsp. <i>akhurstii</i> W14	D. Bowen, University of Wisconsin
TT01	<i>P. luminescens</i> subsp. <i>laumondii</i> TT01	N. Boemare, University of Montpellier
K122	<i>P. luminescens</i> subsp. <i>temperata</i> K122	D. Clarke, University of Bath
ATCC43949	<i>P. asymbiotica</i>	ATCC collection
Lumicin expressing ^a clones from <i>P. luminescens</i> W14		
<i>lum1</i> locus	1AC4, IBC3, 2BH7, 3BD3	Cosmid library
<i>lum2</i> locus	US11, US54	Plasmid library
<i>lum3</i> locus	US25	Plasmid library
<i>lum4</i> locus	US47	Plasmid library

^aAll clones show trypsin-sensitive inhibition of *P. luminescens* K122 and *E. coli*.

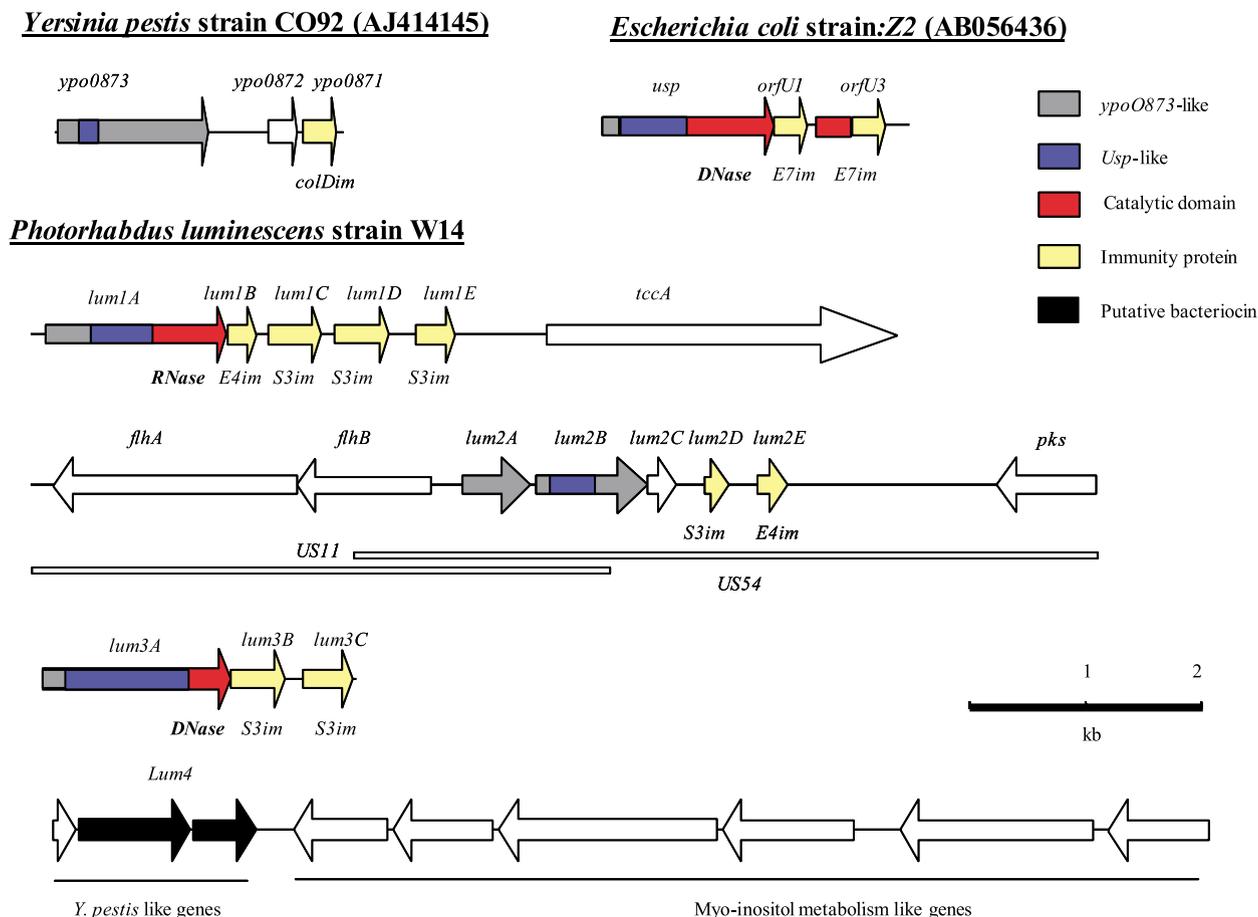


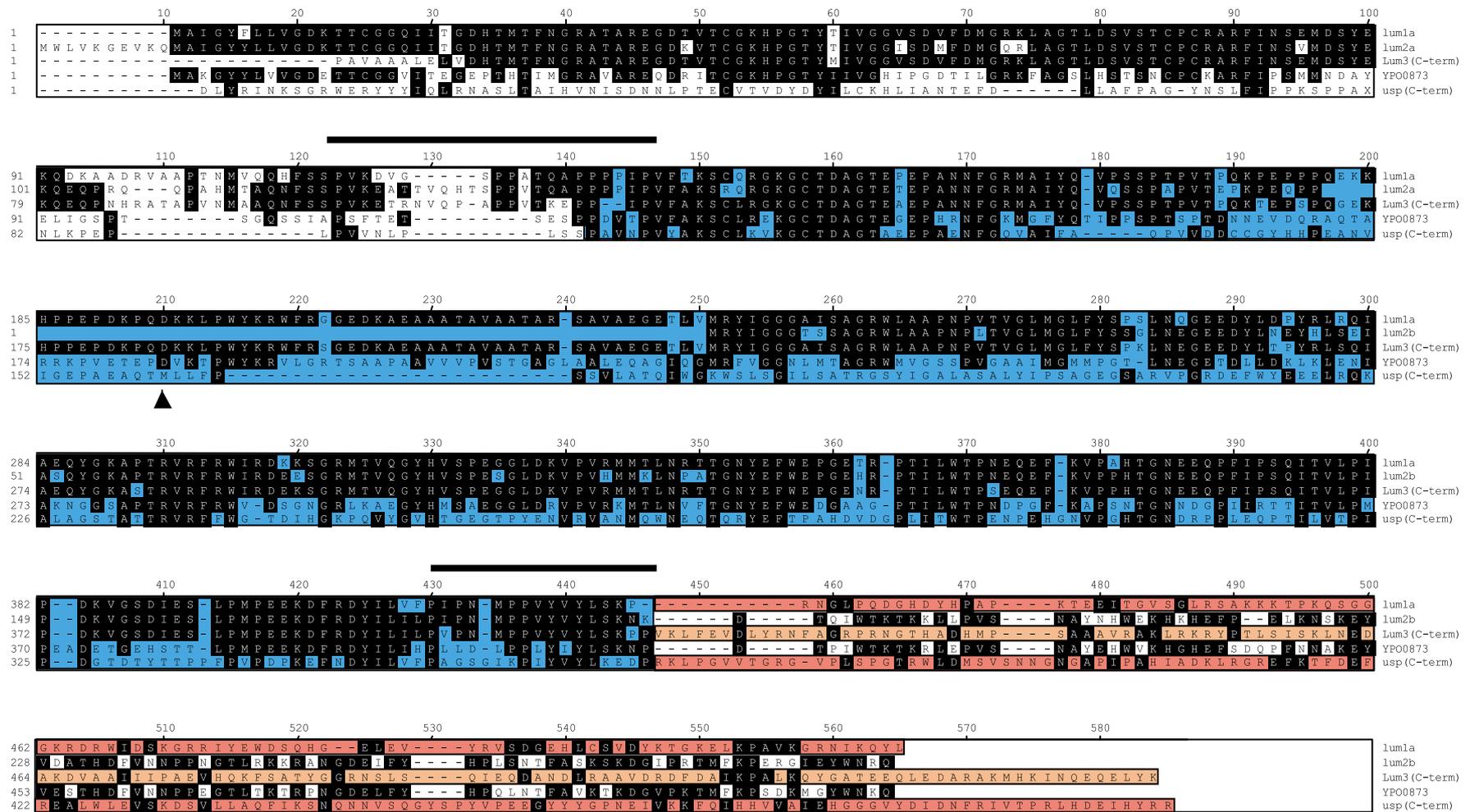
Fig. 2. Genomic organization of the four *lumicin* (*lum*) loci from *P. luminescens* strain W14 compared to a putative bacteriocin locus from *Y. pestis* CO92 and *usp* from uropathogenic *E. coli*. The killer proteins of the *lum* loci encode protein domains with similarity to both the *Y. pestis* and *E. coli* proteins (see legend and text) whilst the multiple immunity proteins are of mixed type. The relative positions of subclones used to test bacteriocin activity (Table 1) are shown.

3.2. Genomic organization and similarity to USP

Four loci with biological activity were identified from both halo assays and the DNA based screens. The predicted proteins encoded by these loci are termed *lumicins* and the loci themselves *lum1*, *lum2*, *lum3* and *lum4*. The *lumicins* are chimeric in nature and share homology with two other putative bacteriocin-encoding *usp* loci from uropathogenic *E. coli* and locus *ypo0873* from *Yersinia pestis* CO92 (Fig. 2). Thus the genomic organization of the *lum* loci is interesting for several reasons. First, they show similarities to *usp* described from a uropathogenic *E. coli* strain suggested to encode proteins putatively involved in uropathogenicity. Our re-analysis of this locus (Fig. 2) suggests that *usp* in fact encodes a bacteriocin killer protein carrying a DNase-like catalytic domain and two E7-like immunity proteins. Second, the *lum* loci also show partial similarities to a novel bacteriocin-like locus from *Y. pestis* CO92 (Fig. 2). This putative bacteriocin-encoding locus predicts a killer protein with no recognizable catalytic domain and two colicin D-like immunity proteins. Third, the killer proteins of the *lumicins* themselves are

fusions of three different domains, ascribed by their similarity to other bacteriocins.

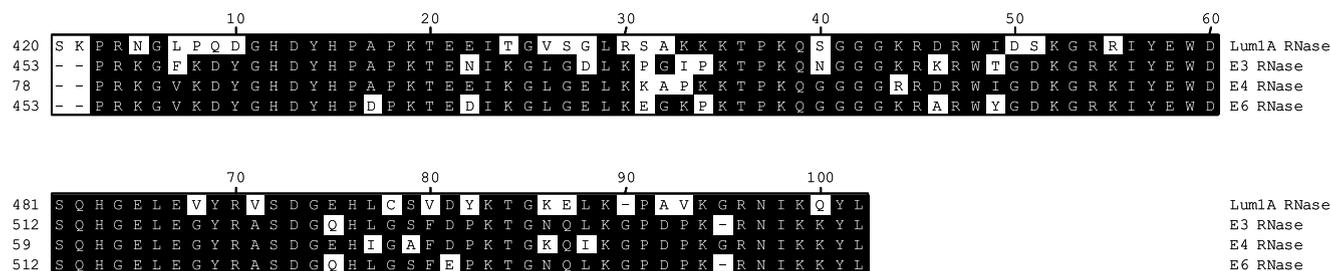
Within these three domains, the central *lumicin* killer protein domain always shows similarity to USP whilst the N- and C-terminal domains are variable (Figs. 2 and 3). The N-termini of the *lumicin* killer proteins show similarities to each other (*Lum1A* and *Lum2A*) and also to the C-terminal domain of *Lum3A* and USP. The C-termini encode putative catalytic domains, predicting RNase (*Lum1A*), DNase (*Lum3A*) or unknown (USP) activities (Fig. 3). Alignment of the predicted RNase domain from the *lum1* encoded killer protein with RNase domains from E3, E4 and E6 shows clear similarity with colicin killer protein RNase-like domains (Fig. 4A). Alignment of the predicted DNase domain from *Lum3* with DNase domains from S1, S2 and S3 pyocins supports the hypothesis that *Lum3* encodes a DNase activity and also supports the concept that USP carries a similar catalytic activity (Fig. 4B). We note that the boundaries between the different domains of the putative killer proteins of *lum1*, *lum2* and *lum3* show regions of a simple proline-rich predicted amino acid (aa) sequence which are similar in other bac-



RNase
 DNase
 Usp like domain
 Proline rich domain boundary

Fig. 3. Predicted amino acid sequence alignment of putative killer proteins from the *Photorhabdus lum1*, *lum2* and *lum3* loci with the two putative bacteriocin-encoding loci from *Y. pestis* (YPO0873) and uropathogenic *E. coli* (*usp*). The killer proteins all carry a central USP-like domain and then show variable C- and N-terminal domains. The C-terminal domains encode predicted RNase or DNase activities (Fig. 4). Note that putative bacteriocin proteins from *lum4* do not show any similarity to known bacteriocin sequences and are therefore not included.

A. RNase Domains



B. DNase Domains

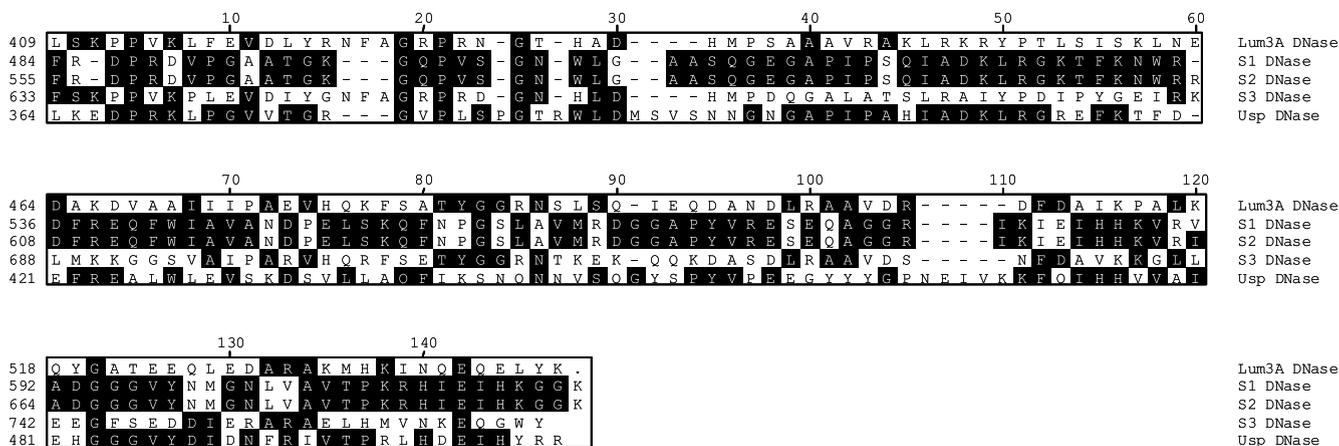


Fig. 4. Predicted amino acid sequence alignments of the *lum1* encoded RNase and *lum3* DNase domains with similar colicin and pyocin domains. A: The strong similarity of the predicted C-terminus of the Lum1A encoded killer protein with E3, E4 and E6 colicin RNase domains supports the hypothesis that *lum1A* encodes RNase activity. B: The similarity of the C-terminus of Lum3A to DNase domains from S1, S2 and S3 pyocins suggests that Lum3A encodes DNase activity.

teriocins (Fig. 3). The predicted ORFs from the fourth locus (termed *lum4*) show no similarity to known or predicted sequences in GenBank but lie next to an operon involved in myoinositol catabolism. We therefore cannot predict any likely mode of action of the predicted Lum4 proteins.

The *lum1*, *lum2* and *lum3* loci are also unusual in the number and organization of predicted immunity proteins that they carry (Fig. 2). The *lum1* locus predicts a colicin E4 immunity protein (Lum1B) of 85 aa downstream from the E4-like C-terminus of the killer protein, as expected. However, subsequently there is also a tandem array of three S3-like immunity proteins (Lum1CDE), of 155, 110 and 113 aa respectively (Fig. 2), whose role is not clear. Multiple immunity proteins are also found in the other *lum* loci, with *lum2* predicting both a single E4-like (85 aa) and an S3-like (153 aa) immunity protein, and *lum3* predicting two S3-like immunity proteins. Sequence comparisons of the two E4-like lumicin immunity proteins show that they are highly related to each other and also similar to E3 and E6 colicin immunity proteins (Fig. 4A). Sequence comparison of the different S3-like lumicin immunity proteins, however, reveals some interesting differences. Thus the S3-like protein from *lum3* and the first S3-

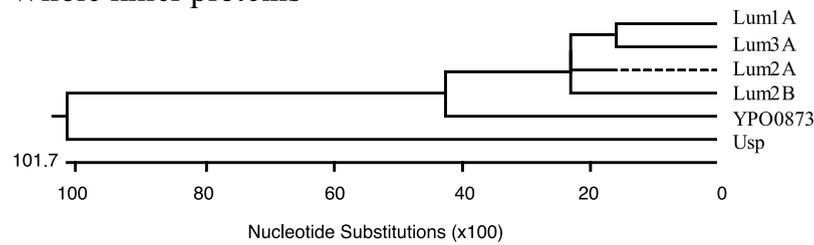
like immunity protein in the *lum1* locus (Lum1C) are of a similar overall predicted length, whereas the subsequent two predicted immunity proteins (Lum1DE) are truncated at their N-termini (alignment not shown). This difference within the S3-like lumicin immunity proteins suggests that they may contain domains dispensable in some contexts. This hypothesis is supported by the observation that predicted immunity proteins from the *usp* locus also share similarity with this class and are of equivalent length to the truncated S3-like lumicins (Fig. 2).

The *lum4* locus is striking in that the putative predicted proteins encoded show no similarity to current sequences in GenBank and do not appear to be related to the other lumicin-encoding loci. We have included this locus with the other lumicins as it encodes a trypsin-inhibitable antibiotic activity.

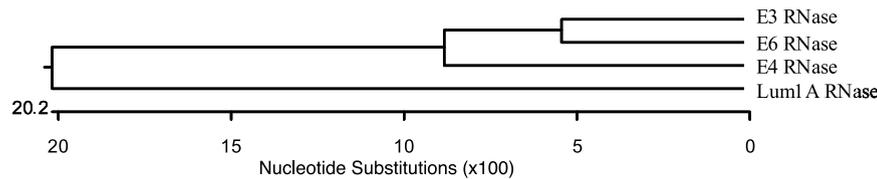
3.3. Phylogenetic relationship with other bacteriocins

Phylogenetic trees derived from the complete ORFs of lumicin killer proteins support their relatedness to USP and YPO0873 (Fig. 5A); however, they suggest overall that the lumicins are more closely related to the putative bacteriocin YPO0873 from *Y. pestis*. Analysis of the rela-

A. Whole killer proteins



B. RNase domains



C. DNase domains

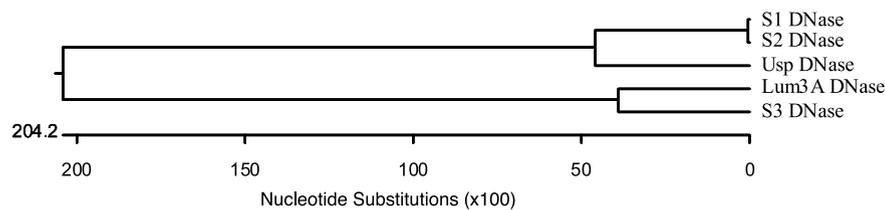


Fig. 5. Killer protein phylogenies based on (A) the complete killer protein ORFs, (B) the predicted RNase domains alone and (C) the DNase domain alone. The phylogeny based on the complete lumicin killer protein ORFs suggests they belong to a single family more distantly related to the putative bacteriocins YPO0873 from *Y. pestis* and *usp* from *E. coli*. Analysis of the RNase domain of Lum1A suggests that it is similar to E4-like RNases. Whilst analysis of the *lum3A* putative DNase suggests that it is related to the pyocin S3 DNase whilst the *usp* putative DNase is more closely related to that from S1 and S2 pyocins.

tionship between the RNase (Fig. 5B) and DNase (Fig. 5C) domains of the lumicin killer proteins supports the notion that they are related to E4-like and S3-like domains respectively. A combined phylogeny of all the different lumicin encoded immunity proteins (Fig. 6) shows that they fall into three broad clades: one S3-like, one E4-like and the other a more poorly defined USP-like clade. The *Y. pestis* immunity protein from YPO0873 does not fall into any of these three clades and appears distantly related.

4. Discussion

Several features of the lumicins are strikingly different from those of the colicins and pyocins, the bacteriocins with which they share most sequence similarity. First, although some of the predicted killer and immunity proteins show similarity to colicins, we have no evidence to suggest that the equivalent genes in *Photobacterium* are plasmid-encoded. Second, *lum2* shows similarity to a putative novel bacteriocin (YPO0873) from *Y. pestis* CO92 (Fig. 2). This putative *Y. pestis* protein has been proposed to belong to a new class of bacteriocins [1] and the equivalent lumicins may therefore represent wider members of this new group. Third, although *lum3* is pyocin S3-like, we

note that S3 is a unique member of the pyocin family with little homology to other pyocins, carrying no H-N-H motif within the predicted DNase domain [28]. Thus *lum3* shows homology to a very different member of this family. Fourth, the killer proteins of the lumicins are 'fusion' proteins of multiple domains predicted from their presence in other bacteriocins. These fusions include a central domain with similarity to the predicted USP protein (Fig. 3), a protein predicted from the gene *usp* identified in a collection of uropathogenic *E. coli* [22]. Finally, the lumicin loci also carry multiple mixed-type immunity proteins (Fig. 2). Although this is not unprecedented [1], the prediction of four immunity proteins (three with similarity to pyocin S3 and one with similarity to colicin E4 immunity proteins) from the *lum1* locus may suggest a novel breadth of immunity protein protection afforded by this locus.

The chimeric nature of some bacteriocin loci has been noted previously, for example the *klebicin B* gene cluster has regions similar to pore-forming colicins, nuclease pyocins and other colicins [29]. However, the chimeric nature of the lumicin killer proteins is not only striking but also raises interesting questions both for their modes of action and their relatedness to sequences in other bacteria (Fig. 5). Although the C-termini of the killer proteins clearly predict a potential mode of action for each protein, namely

Immunity proteins

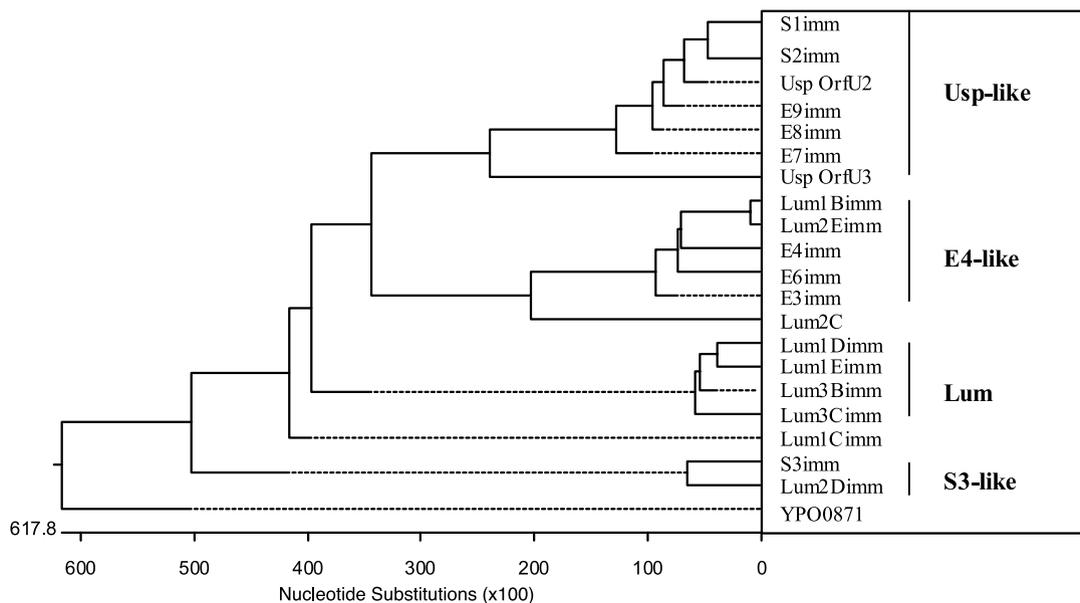


Fig. 6. Immunity protein phylogeny based on all the predicted immunity proteins from the *lum1*, *lum2* and *lum3* loci. This tree supports the concept that the lumicin loci encode multiple dual type immunity proteins each of which is either S3-like, E4-like or *usp*-like (three clades labelled). Note that the putative *Y. pestis* immunity protein from locus YPO0873 appears unrelated to any of these clades.

DNase activity for *lum2* and *lum3*, and RNase activity for *lum1* (Fig. 4), the remainder of the predicted proteins give little indication as to either the predicted target cell receptor or whether each fusion carries more than one killer function. Thus the USP-like sequence (or the novel N-terminus) may target a previously undocumented receptor on the target cell surface, or the sequence shared by the lumicins and USP may itself encode an unknown killer function. This latter possibility is supported by the observation that subclone US11 from the *lum3* locus is associated with a halo phenotype but does not contain the putative C-terminal catalytic domain (Fig. 2).

The lumicin loci are also notable in that they bear multiple dual-type immunity proteins broadly classified as either S3-like, E4-like or USP-like (Fig. 6). Immunity proteins bind with very high affinity to the cytotoxic killer proteins [2]. Therefore the prediction that *lum1* carries both S3 pyocin-like and E4 colicin-like immunity proteins suggests that they can protect against a dual mode of killer protein action. As the E4-like immunity protein is encoded immediately downstream from an E4 RNase-like domain (Fig. 2), it seems reasonable to assume that these are cognate partners. In the absence of any other clear catalytic activity domains in the *lum1* killer protein, we can only speculate as to the likely partners of the three S3-like immunity proteins. However, the presence of these three additional immunity proteins raises the formal possibility that they could protect against the cytotoxic effects of undocumented killer domains in the *lum1* killer protein or indeed bacteriocins from competing strains of *Photobacterium*. Such cross-protection would give insect or nem-

atode colonizing strains of *Photobacterium* unique competitive ability.

Although we have no data to support the likely role of the lumicin loci in the *Photobacterium* life cycle, we can speculate as to when and where bacteriocin activity may be required. Given the strict association between a single strain of *Photobacterium* and its carrier nematode, bacteriocin activity could be useful for maintaining a monoculture of a specific strain in the gut of the nematode symbiont itself. Following release of *Photobacterium* from the nematode gut into the insect blood system and destruction of the insect's gut, the bacteria would need to kill other invading bacteria. Such bacteria could be external in origin (other bacteria carried by other entomopathogenic nematodes and soil borne bacteria) or internal (gut microflora released from the gut after its destruction during the early stages of infection) [12]. Whatever the source of bacterial competitors, the observation that the lumicins can kill such distantly related bacteria as *E. coli*, and the fact that they carry multiple killer and immunity proteins, suggest that they may help in killing or counteracting the competitive effect of a wide range of invading bacteria.

Finally, recent studies have shown that the genes encoding the putative protein USP in *E. coli* isolates are present within uropathogenic strains, suggesting that they may play an undocumented role in disease [22–24]. The results described here, which show that the predicted *E. coli* USP protein is a putative bacteriocin with a DNase catalytic domain and the homology of USP to elements of the lumicins, support an alternative hypothesis that USP is itself a bacteriocin [25]. This raises the interesting possibil-

ity that both USP and the lumicins may form a novel group of chromosomally encoded bacteriocins responsible for enhancing competitive infectivity, in the human urinary tract and the insect blood system, respectively.

Acknowledgements

We thank the donors listed in Table 1 for kind provision of strains. Supported by grants from the BBSRC initiative Exploiting Genomics and by a Royal Society Merit Award to R.ff-C.

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