Subspecies diversity in bacteriocin production by intestinal *Lactobacillus salivarius* strains

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Summary

A recent comparative genomic hybridisation study in our laboratory revealed considerable plasticity within the bacteriocin locus of gastrointestinal strains of *Lactobacillus salivarius*. Most notably these analyses led to the identification of two novel unmodified bacteriocins salivaricin L and salivaricin T produced by the neonatal isolate *L. salivarius* DPC6488 with immunity, regulatory and export systems analogous to those of abp118, a two-component bacteriocin produced by the well characterized reference strain *L. salivarius* UCC118. In this addendum we discuss the intraspecific diversity of our seven bacteriocin-producing *L. salivarius* isolates on a genome-wide level, and more specifically, with respect to their salivaricin loci.

Introduction

In a recent comparative study, we investigated the diversity of the bacteriocin loci of seven *Lactobacillus salivarius* isolates of human and porcine intestinal origin isolated in our laboratory.[1] The bacteriocin loci of the respective strains were compared with that of the *L. salivarius* UCC118, a probiotic candidate which produces the two-component class IIb bacteriocin abp118.[2] Notably, the probiotic efficacy of this bacteriocin has been reported by Corr and coworkers.[3] Specifically, this study demonstrated that abp118 production was directly responsible for the inhibition of *Listeria monocytogenes* in a murine infection model following oral administration of *L. salivarius* UCC118, thereby corroborating the role of bacteriocin production in probiosis.[3] Furthermore, the bacteriocin-mediated ability of *L. salivarius* UCC118 to influence the composition of the gut microbiota of diet induced obese (DIO) mice was recently demonstrated.[4] Interestingly, abp118 did not impact total faecal bacterial numbers. Rather, an increase in the relative proportions of *Bacteroidetes* and *Proteobacteria* and a decrease in *Actinobacteria* were characteristic of the gut microbiota of DIO mice administered the abp118-producing probiotic in comparison to those fed a bacteriocin-deficient derivative of *L. salivarius* UCC118.

Possession of the genetic determinants responsible for the production of such two component class II bacteriocins is widespread amongst *L. salivarius* isolates of intestinal origin.[5-8] In addition to the bacteriocin structural genes, the abp118 locus is comprised of genes involved in bacteriocin immunity (*abp118IM*), regulation (*abp118IP*, *abp118K*, *abp118R*) and transport (*abp118T* and *abp118D*), all required for efficient bacteriocin production and protection of the producing strain.[2] In our study, microarray-based comparative genomic hybridization (CGH) analyses based on the genome of *L. salivarius* UCC118 revealed that the abp118-related genes were

conserved in all test strains with the exception of one porcine isolate, *L. salivarius* DPC6502. The four remaining isolates of porcine origin had previously been shown to produce salivaricin P, a natural variant of abp118.[5] The observation that the genes involved in bacteriocin transport were absent in the human isolate *L. salivarius* DPC6196, most likely explains the bacteriocin negative phenotype of this strain as the gene cluster was otherwise highly conserved. Although genes involved in abp118 regulation and transport were well conserved within the second strain of human origin, *L. salivarius* DPC6488, considerable diversity was evident with respect to the structural genes. Indeed, four open reading frames (ORFs) potentially encoding putative bacteriocin prepeptides were identified in the bacteriocin locus of this strain. Three of these were found to contribute to the production of two novel bacteriocins designated salivaricin T and salivaricin L, while the fourth encoded an inactive homologue of salivaricin B. Like abp118, salivaricin T is a two-component bacteriocin. However, the mature peptides of this narrow spectrum bacteriocin did not resemble those of abp118 but rather, thermophilin 13, a bacteriocin produced by *Streptococcus themophilus*.[9] In contrast, salivaricin L is a one peptide bacteriocin of the class IId variety which exhibited anti-*Listeria* activity. Overall, these analyses exposed an unprecedented level of versatility within the bacteriocin loci of the *L. salivarius* candidate probiotics.

Plasticity of seven *L. salivarius* genomes of human and porcine origin

In this manuscript, an overview of the genome as a whole revealed that this plasticity was not exclusive to the bacteriocin locus of *L. salivarius* UCC118 but was reflected across 23 hyper-variable clusters within the test strains (Fig. 1, Table 1). Indeed, just 72% of the *L. salivarius* UCC118-specific features represented on the array were

common to all seven test strains and, interestingly, 12% of features were exclusive to strain UCC118. The genome of *L. salivarius* UCC118 is comprised of a circular chromosome of 1.8 MB, complemented by a megaplasmid, pMP118 (242 kb; on which the genetic determinants for abp118 are located) and two smaller plasmids, pSF118-20 and pSF118-44.[10] Our results indicated that the human isolate deficient for bacteriocin activity *L. salivarius* DPC6196 possessed the greatest percentage (88%) of UCC118-specific genes, while *L. salivarius* DPC6488, which produces the novel salivaricins T and L, harboured 84%. The porcine intestinal isolate *L. salivarius* DPC6502 displayed the greatest divergence, with 78% conservation of the UCC118 gene content. The remaining porcine isolates, *L. salivarius* DPC6005, DPC6027, DPC6189 and 7.3, displayed between 79% and 84% conservation. These findings were largely consistent with a previous survey of the genomic diversity of 33 *L. salivarius* isolates of various origins.[11] We identified ninety six genes which represented the regions of greatest divergence, i.e. present in strain UCC118 but absent from all seven test isolates. These were typically components of mobile DNA elements such as prophage and plasmid-associated genes, as summarised here.

Regions of greatest divergence

Neither of two complete prophage of *L. salivarius* UCC118, Sal1 and Sal2 (corresponding to hyper-variable regions HV 7 and HV 3 respectively), were fully conserved in any of the seven test strains. With respect to the plasmid content, the conservation of LSL_1739 (*repA*) indicated the presence of *repA*-type megaplasmids in all strains. The megaplasmid encoded choloylglycine hydrolase (LSL_1801), primarily responsible for the bile-salt hydrolase activity of *L. salivarius* UCC118,[12] was also well conserved in all strains while hypothetical proteins, pseudogenes and

transposases were largely responsible for diversity with respect to pMP118-related genes in the test strains. Notably, a remnant of a conjugal plasmid transfer locus in pMP118 (HV 20) was not conserved in either of the human test strains nor the porcine isolate *L. salivarius* DPC6502. Although genes associated with the smallest replicon of strain UCC118, pSF118-20, were generally absent from all test strains, *L. salivarius* DPC6488 DNA hybridized to probes corresponding to the replication proteins of both of the smaller replicons (LSL_1965 and LSL_2000), indicating the presence of somewhat related plasmids in this strain. The human isolate *L. salivarius* DPC6196, was the only strain in which the genes of pSF118-44 were almost completely conserved. LSL_2000 was also conserved in strain DPC6189 indicating that this strain may also harbour a pSF118-44-like plasmid. However, the genes associated with this replicon were absent from all other test strains of porcine origin.

Regions distinguishing isolates of human and porcine origin

Interestingly, a hierarchical tree which was generated on the basis of the variability of the data, sub-grouped the respective test strains of human and porcine origin (Fig. 2), with the latter group displaying greatest diversity with respect to the human-associated *L. salivarius* UCC118. Although, it may be possible that this is a result of the small number of test strains investigated in this instance or perhaps due to an imbalance of strains from these individual hosts. Gene clusters to which this distinction was attributed were both chromosomally and megaplasmid located and often associated with fitness, niche adaptation, and potentially the probiotic functionality of the strains (Fig. 1, Table 1). It is possible, for example, that the absence of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) - associated genes represented by hyper-variable region 1 (HV 1) and genes associated

with a type I restriction–modification system (HV 8), features which confer resistance to foreign DNA elements, in all of the porcine test strains may render these isolates susceptible to phage attack within the GIT.

Protection and stress tolerance as well as adhesion and *in vivo* persistence are also among the many benefits associated with exopolysaccharide (EPS) production which may be important factors for colonization and survival within the GIT.[13] Both EPS clusters 1 (HV 10) and 2 (HV 17) of strain UCC118 were identified as strain specific traits. Although many of the genes associated with cluster 2 were not well conserved in any of the test strains, cluster 1 was clearly absent from all porcine derived isolates.

The presence of multiple mannose phosphotrasferase systems (pts) has been associated with enhanced metabolic versatility of microorganisms, as well as horizontal gene transfer events.[14] Therefore, it is notable that two of the four mannose pts systems of *L. salivarius* UCC118 (HV 19 and 22) were also absent in all of the porcine derived test strains.

Bacteriocin loci of porcine-derived test strains

Despite the absence of the aforementioned features, the porcine isolates included in this study were originally recovered from intestinal origins as a consequence of their associated antimicrobial activity.[15, 16] The production of organic acids, hydrogen peroxide and bacteriocins may all contribute to this phenotype, however, the widespread distribution of the salivaricin P locus in *L. salivarius* isolates of porcine origin may be indicative of its importance for colonization of the porcine GIT. Further substantiating this hypothesis, findings by Walsh et al., (2008) revealed that the salivaricin P-producing component *L. salivarius* DPC6005 predominated within

the porcine ileum over four counterparts orally administered as a probiotic formulation.[17] This strain was among four porcine intestinal isolates included in our study, *L. salivarius* DPC6005, DPC6027, DPC6189 and 7.3, which were previously shown to produce this natural variant of abp118. The homology of the individual salivaricin P structural genes sln1 and sln2 of each of these strains was previously established.[5] This conservation is also evident from our corresponding CGH data, however, diversity was evident elsewhere within the salivaricin P loci of each of the producing strains. This diversity, coupled with the revelation of novelties within the corresponding gene cluster of *L. salivarius* DPC6488, encouraged further analysis of the salivaricin P gene cluster, as described in detail below.

A representative salivaricin P gene cluster, consisting of a contiguous sequence of 13,256 nucleotides, was amplified and sequenced using *L. salivarius* DPC6005 template DNA and oligonucleotide primers designed based on the sequence of the abp118 locus. Nineteen putative ORFs were identified, which were arranged in a similar manner to the genetic determinants of the abp118 and salivaricin T/L loci of *L. salivarius* UCC118 and *L. salivarius* DPC6488, respectively (graphically represented in Fig. 2). An alignment revealed that the 10.7 kb abp118 locus (accession number AF408405)[2] shared 90% similarity with the salivaricin P sequence of strain DPC6005 and functions were assigned to the products encoded by eight putative ORFs of the salivaricin P cluster based on homology with their UCC118 counterparts (Table 2). In agreement with our data, Barrett and co-workers previously revealed that the structural genes encoding the two component salivaricin P peptides, *sln1* and *sln2*, share 98% and 97% identity with *abp118α* and *abp118β*, respectively, which corresponds to 100% and 95% identity, respectively, between the corresponding mature bacteriocin sequences.[5] The deduced product of a single ORF upstream of the

structural genes, ORF4, displayed similarity to the bacteriocin-like prepeptide products of both of the UCC118 associated genes LSL_1918 and LSL_1920 (95% and 70%, respectively), which may be indicative of a gene duplication event at this site. The deduced protein encoded by ORF3 exhibited 94% identity with the salivaricin B bacteriocin precursor peptide, produced by *L. salivarius* M6, and its inactive UCC118 (LSL_1921) and DPC6488-associated homologues.[2, 18] This peptide was not detected during the purification of the antimicrobial components of *L. salivarius* DPC6005 and thus, is also considered inactive in this strain.[5] Immediately downstream of the structural genes are two putative ORFs potentially encoding immunity (ORF7) and induction (ORF8) proteins which share 80% and 60% identity with the analogous proteins encoded by UCC118, respectively. The similarity of the putative induction peptide of the salivaricin P regulatory system lies mainly within the double-glycine leader sequence (17 amino acids (aa)), as the mature peptides (22 aa) share just 40% identity. It is, thus, not surprising that the histidine kinase encoded by *slnK* displayed just 69% homology with its abp118 counterpart, AbpK. Indeed, these two proteins exhibited greatest diversity in the N-terminal domain responsible for sensing the cognate induction peptide. Although SlnK shares 93% similarity with AbpK of *L. salivarius* DSM20555 (accession number EEJ73430), DSM20555 does not possess an anti-*Listeria* phenotype.[6] The proteins encoded by the genes adjacent to s*lnK* shared greater than 95% homology with the response regulator and the gene products involved in transport of abp118 (Table 2). The sequence and putative ORFs downstream of the designated transport system exhibit little similarity with the abp118 locus. However, the proteins encoded by ORF15 and ORF16 display similarity to the hypothetical proteins encoded by LSL_1832 and LSL_1831, two genes located approximately 74 kb upstream of the abp118 gene cluster on pMP118,

perhaps indicating the occurrence of a recombination event. Inverted repeat sequences typical of rho-independent transcription termination signals were identified at three locations. Those downstream of ORF2 and ORF18, with calculated ΔG of -20.10 kcal/mol and -19.50 kcal/mol,[19] respectively, may represent the beginning and end of the salivaricin P operon, respectively. The third possible rho-independent terminator was identified downstream of *sln2* (ΔG of -22.10 kcal/mol) and may serve as an attenuator to ensure a higher transcription level of the bacteriocin structural genes than the ORFs downstream, a feature frequently observed in the genetic loci of regulated bacteriocins.[9, 20, 21] Although novel bacteriocin genes or remnants thereof were not identified, the sequence data of the salivaricin P locus of DPC6005 strongly correlated with our CGH data.

Considering the bacteriocin-mediated ability of *L. salivarius* to modulate the gut microbiota, in particular with respect to providing protection against *Listeria* infection, this hitherto unknown level of intra-species diversity with respect to bacteriocin production by intestinal *L. salivarius* isolates is of considerable significance. In addition, the consequence of this diversity is probably that strains can adapt to very different gastrointestinal environments as evidenced by the delineation between human and porcine strains in this study.

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Table 1. Composition of hyper-variable regions within L. salivarius species relative to L. salivarius UCC118

HV	Proposed function		Size (kb)	Genes	GC %[a]
1	CRISPR genes		7.786	LSL_0098-LSL_0100	30
2	Carbohydrate metabolism		5.385	LSL_0142-LSL_0148	33
3	Prophage Sal2		39.622	LSL_0236-LSL_0305	33
4	Hypothetical proteins		6.135	LSL_0349-LSL_0352	31
5	Hypothetical proteins		1.816	LSL_0519-LSL_0521	26
6	Transposases		1.583	LSL_0585-LSL_0586	32
7	Prophage Sal1		47.905	LSL_0729-LSL_0805	32
8	Type I restriction-modification system		9.73	LSL_0915-LSL_1920	30
9	Hypothetical proteins		2.314	LSL_0942-LSL_0945	30
10	EPS cluster 1		23.521	LSL_0975-LSL_0997	32
11	Hypothetical proteins		15.795	LSL_1012-LSL_1024	31
12	Prophage Sal4		8.906	LSL_1189-LSL_1205	31
13	Mucus-binding proteins		7.893	LSL_1334-LSL_1340	32
14	Hypothetical proteins		23.395	LSL_1380-LSL_1401	35
15	Hypothetical proteins		4.597	LSL_1492-LSL_1497	30
16	Hypothetical proteins		14.441	LSL_1522-LSL_1527	28
17	EPS cluster 2		34.726	LSL_1546-LSL_1573	30
18	Prophage Sal3		10.017	LSL_1648-LSL_1666	31
19	Mannose PTS system		8.253	LSL_1708-LSL_1716	32
20	Conjugation region		67.138	LSL_1808-LSL_1869	32
21	Bacteriocin locus		11.008	LSL_1906-LSL_1924	30
22	Mannose pts system		4.609	LSL_1949-LSL_1955	32
23	Small plasmids	pSF118-20	20.417	LSL_1960-LSL_1986	39
		pSF118-44	44.013	LSL_1987-LSL_2037	39

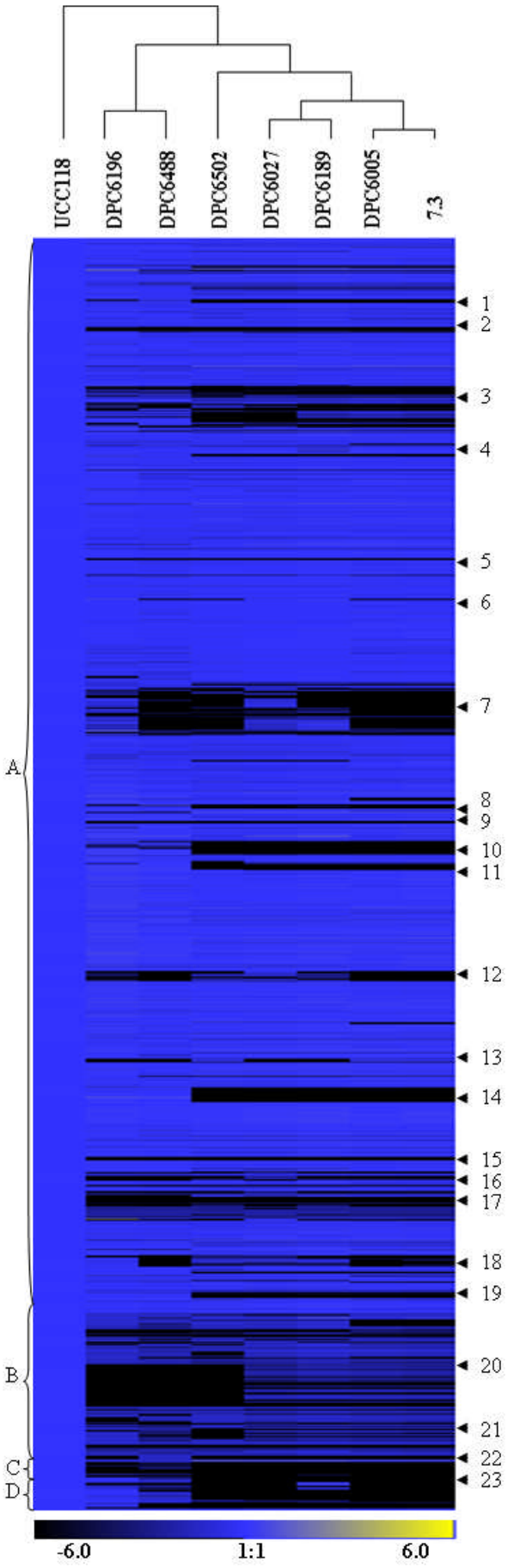
[a] The GC content of the chromosome of *L. salivarius* UCC118 is 32 %

Table 2. Proteins encoded by the salivaricin P locus and similarity to their homologues

ORF (gene)	Size (aa)	Function	Homologue	Identity (%)[a]	Reference
ORF 1	65	Conserved hypothetical protein	Conserved hypothetical protein of *L. salivarius* DSM20555	95 [62/65]	EEJ73426[b]
ORF 2	87	Conserved hypothetical protein	Conserved hypothetical protein of *L. salivarius* DSM20555	98 [86/87]	EEJ73427[b]
ORF 3	57	Bacteriocin-like prepeptide	Salivaricin B prepeptide	94 [54/57]	(5)
ORF 4	85	Bacteriocin-like prepeptide	LSL_1918 of *L. salivarius* UCC118	95 [81/85]	(6)
ORF 5 (*sln1*)	64	Salivaricin P prepeptide Sln1	Abp118 bacteriocin alpha prepeptide (LSL_1917)	100 [64/64]	(13)
ORF 6 (*sln2*)	68	Salivaricin P prepeptide Sln2	Abp118 bacteriocin beta prepeptide (LSL_1916)	97 [66/68]	(13)
ORF 7 (*slnIM*)	44	Putative salivaricin P immunity protein	Abp118 IM (LSL_1915) of *L. salivarius* UCC118	80 [33/41]	(13)
ORF 8 (*slnIP*)	39	Putative salivaricin P induction peptide	Abp118 IP (LSL_1914) of *L. salivarius* UCC118	60 [24/40]	(13)
ORF 9 (*slnK*)	430	Sensory transduction histidine kinasse	AbpK of *L. salivarius* DSM20555	93 [401/430]	EEJ73430[b]
ORF 10 (*slnR*)	266	Response regulator	AbpR (LSL_1912) of *L. salivarius* UCC118	96 [255/264]	(13)
ORF 11	79	Hypothetical membrane spanning protein	LSL_1911 of *L. salivarius* UCC118	88 [70/79]	(6)
ORF 12	65	Hypothetical protein	Hypothetical protein HMPREFOS45_1706 of *L. salivarius* DSM20555	92 [60/65]	EEJ73433[b]
ORF 13 (*slnT*)	719	Salivaricin P ABC-transporter protein	AbpT (LSL_1910) of *L. salivarius* UCC118	97 [698/719]	(13)
ORF 14 (*slnD*)	382	Salivaricin P export accessory protein	AbpD (LSL_1909) of *L. salivarius* UCC118	95 [365/381]	(13)
ORF 15	73	Hypothetical protein	LSL_1832 of *L. salivarius* UCC118	87 [64/73]	(6)
ORF 16	134	Hypothetical protein	LSL_1831 of *L. salivarius* UCC118	82 [110/133]	(6)
ORF 17	209	Hypothetical protein	no homologues		
ORF 18	315	Hypothetical protein	no homologues		
ORF 19	106	Conserved hypothertical protein	Conserved hypothertical protein *L. salivarius* DSM20555	95 [84/88]	EEJ73436[b]

[a] Percentage identity was determined using BLAST

[b] Accession number of sequence directly submitted to EMBL Database


UCC118
DPC6196
DPC6488
DPC6502
DPC6027
DPC6189
DPC6005
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A
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C
D
-6.0
1:1
6.0

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Fig. 1. Analysis of genomic diversity of *L. salivarius* test strains with respect to *L. salivarius* UCC118 by CGH. Replicons are in the order of chromosome (A), pMP118 (B), pSF118-20 (C) and pSF118-44 (D). Black, blue and yellow regions represent absence, conservation or overrepresentation of CDS, respectively, corresponding to the colour legend. Numbers 1 to 23 represent hyper-variable regions within the *L. salivarius* species, as outlined in Table 1.

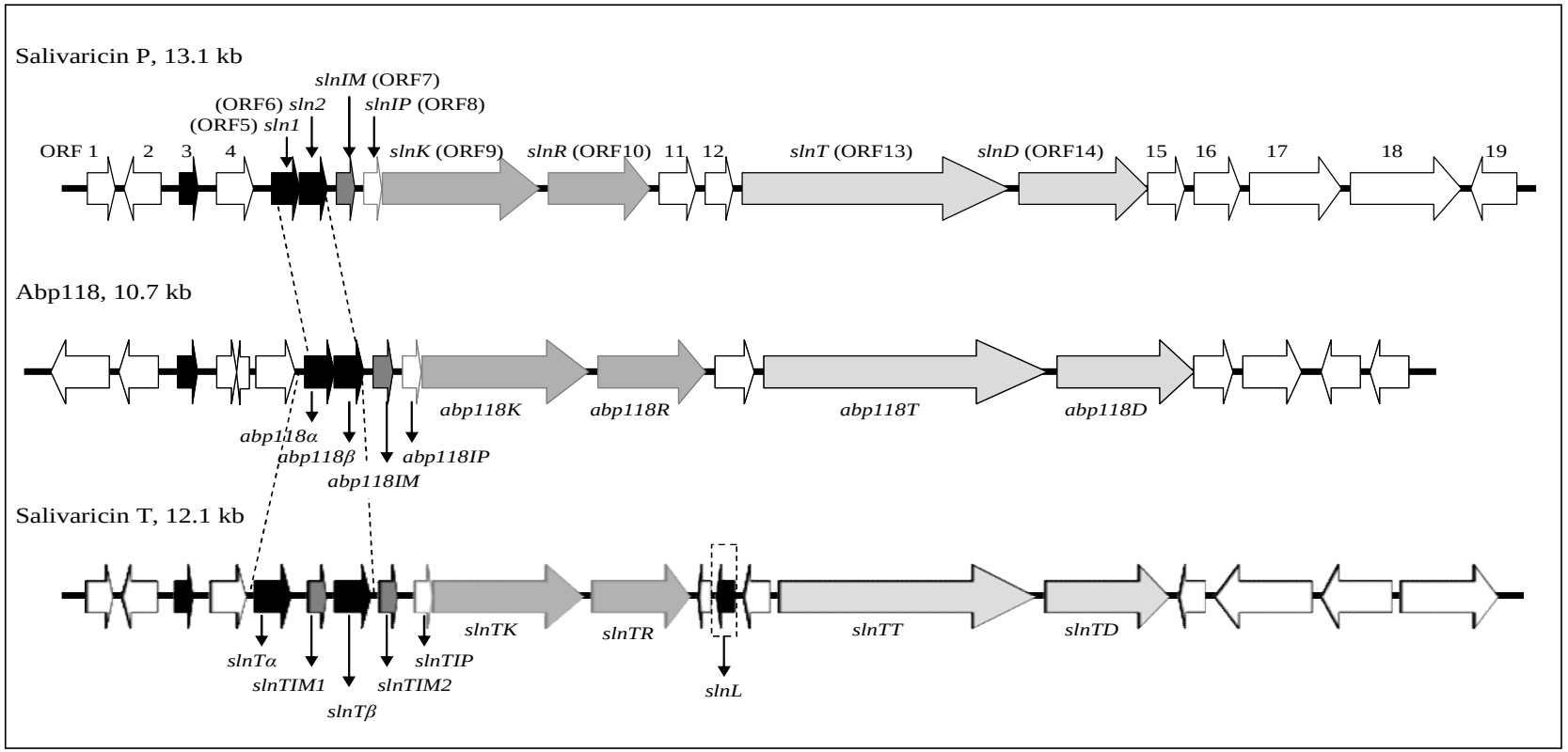

Fig. 2. Comparative representation of the salivaricin P gene cluster with that of abp118 and salivaricin T/L. Black and charcoal arrows indicate bacteriocin structural and predicted immunity genes, respectively, while genes involved in regulation and transport are indicated by grey and

those encoding hypothetical proteins by white arrows. The similarity of the putative protein products encoded by the respective gene clusters are outlined in Table 2.