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Genome analysis of two virulent *Streptococcus thermophilus* phages isolated in Argentina

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ABSTRACT

Two *Streptococcus thermophilus* phages (ALQ13.2 and ϕAbc2) were previously isolated from breakdowns of cheese manufacture in Argentina. Complete nucleotide sequence analysis indicated that both phages contained linear double-stranded DNA: 35,525 bp in length for the *pac*-type phage ALQ13.2 and 34,882 bp for the *cos*-type phage ϕAbc2. Forty-four and 48 open reading frames (ORF) were identified for ALQ13.2 and ϕAbc2, respectively. Comparative genomic analysis showed that these isolates shared many similarities with the eight previously studied *cos*- and *pac*-phages infecting different *S. thermophilus* strains. In particular, part of the ϕAbc2 genome was highly similar to a region of phage 7201, which was thought to be unique to this latter phage. Protein analysis of the *pac*-phage ALQ13.2 using SDS polyacrylamide gel electrophoresis (SDS-PAGE) identified three major proteins and seven minor proteins. Parallel structural proteome analysis of ϕAbc2 revealed seven protein bands, two of which were related to major structural proteins, as expected for a *cos*-type phage. Similarities to other *S. thermophilus* phages suggest that the streptococcal phage diversity is not extensive in worldwide dairy factories possibly because related high-performing bacterial strains are used in starter cultures.

1. Introduction

Streptococcus thermophilus is a lactic acid bacterium used extensively in thermophilic commercial starter cultures for the large-scale manufacture of variety of cheeses and yogurts (Reinheimer et al., 1997; Andrighetto et al., 2002). Starter culture performance can be compromised by virulent phage infections leading to fermentation failures or low quality products. Since vast quantities of milk are transformed daily to produce these fermented foods, phage infection represents a significant risk and their population must be kept under control and at a low level on a day-to-day basis (Moineau and Lévesque, 2005). A plethora of strategies have been designed to achieve tight control of dairy phages and they include sophisticated strain rotation schemes, phage resistance mechanisms, and sanitation. These methods have significantly reduced the number of starter culture malfunctions. Nonetheless, new dairy phages continue to emerge regularly in cheese factories, posing a risk to the industry.

Our knowledge of the *S. thermophilus* phage population has increased considerably in the past decade. First, it was found that all known *S. thermophilus* phages have the same general characteristics. They have a double-stranded DNA genome (30-kb to 45-kb) packed into an icosaedric capsid (~60 nm in diameter) connected to a long non-contractile tail (200 to 300 nm). Accordingly, they belong to the *Siphoviridae* family of the *Caudovirales* order (Le Marrec et al., 1997). Prior to the availability of genome sequencing data, these streptococcal phages were further classified into two groups based on their DNA packaging mechanisms (*cos* or *pac*). The characterization of *S. thermophilus* phage genomes led to a more accurate assessment of their natural diversity (Lévesque et al., 2005). For example, comparative genome analyses suggested that all *S. thermophilus* phages belong to one polythetic species of temperate/lytic phages (Brüssow and Desiere, 2001). Despite the *cos*- and *pac*-distinction, further comparisons demonstrated that these genomes are similarly organized into distinct modular regions. In particular, the genes coding for DNA replication and host lysis are highly conserved. These studies also led to a better assessment of their origins, relationships with other phages, and mechanisms responsible for their diversity. The latter is driven by point mutations, gene disruption, and recombination events within functional modules. This genomic diversity is likely a way for the phages to adapt to new host environments, including to natural resistance mechanisms such as CRISPR systems found in *S. thermophilus* cells (Deveau et al., 2008).

The above conclusions were mainly based on the analyses of eight complete phage genomes available in public databases and representing the two recognized *cos* and *pac* groups of *S. thermophilus* phages.

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The complete genome sequences for four *cos*-type phages include the virulent phages DT1 (Tremblay and Moineau, 1999), 7201 (Le Marrec et al., 1997), ϕSfi19 (Lucchini et al., 1998), as well as the temperate phage ϕSfi21 (Desiere et al., 1999). The complete genome sequences for four *pac*-type *S. thermophilus* phages include the temperate phage ϕO1205 (Stanley et al., 1997) and the virulent phages ϕSfi11 (Lucchini et al., 1998), 2972 (Lévesque et al., 2005), and 858 (Deveau et al., 2008). Six of these 8 phages were isolated from failed yogurt manufactures in France (ϕSfi19, ϕSfi21, ϕO1205, ϕSfi11, 2972 and 858) and one from the Netherlands (phage 7201). Phage DT1 was isolated from Canadian cheese whey.

Argentina also has a long tradition in cheese and yogurt manufacture (Quiberoni et al., 2006; Reinheimer et al., 1997) and many virulent *S. thermophilus* bacteriophages have been isolated from breakdowns of these dairy processes (Suárez et al., 2002). Since the majority of *S. thermophilus* phages analyzed thus far have been isolated from geographically close or related regions, Brüssow et al. (1998) suggested that a broader coverage of phages from countries with strong dairy traditions was needed to help establish a correlation between geographical origin and phage diversity.

We report here the complete genomic sequence of two dairy phages from South America namely, the virulent *S. thermophilus* phages ALQ13.2 (*pac*-type) and ϕAbc2 (*cos*-type), isolated from failed Cremoso cheese in Argentina (Quiberoni et al., 2006; Suárez et al., 2002). Our analyses suggest that the *S. thermophilus* phage population is more homogeneous than other groups of dairy phages.

2. Materials and methods

2.1. Bacterial strains and phages

S. thermophilus phages ALQ13.2 (Quiberoni et al., 2006) and ϕAbc2 (Suárez et al., 2002) were previously isolated from failed Cremoso cheese manufactures in Argentina. The host strains of phages ALQ13.2 and ϕAbc2 are *S. thermophilus* ST13.2 (Quiberoni et al., 2006) and Abc2 (Suárez et al., 2002), respectively. They were isolated from commercial starter cultures used for the industrial manufacture of Argentinean Cremoso cheese. These bacterial strains were grown at 42 °C in Elliker broth (Biokar) or in M17 broth (Difco) supplemented with 0.5% (w/v) lactose (LM17). Bacterial strains and phages are maintained at the INLAIN Collection (Argentina) and at the Félix d'Hérelle Reference Center for Bacterial Viruses (Canada, http://www.phage.ulaval.ca) as frozen stocks in LM17 or Elliker broth using a 15% (v/v) glycerol cryoprotectant. Phage propagation was carried out as previously described by Jarvis (1978) and the lysates were centrifuged, filtered (0.45 µm pore diameter filter, Millipore) and kept at 4 °C until use.

The host range of phages ALQ13.2 and ϕAbc2 was carried out by the spot test as previously described (Svensson and Christiansson, 1991), while the burst sizes were performed according to Suárez et al. (2002).

2.2. Phage purification

Phages were purified by ultracentrifugation using two consecutive CsCl gradients. One liter of phage lysate was concentrated with 10% polyethyleneglycol (PEG) (Jarvis, 1978, 1984). The PEG-concentrated phages were subject to ultracentrifugation using a Beckman SW41 Ti rotor (35,000 rpm, 3 h at 20 °C). A second ultracentrifugation was performed using a Beckman NVT65 rotor (60,000 rpm, 18 h at 20 °C). The purified phages (approximately 10^{11} PFU/ml) were then dialyzed against phage buffer (Spectra®/Por membranes, Spectrum® Laboratories, Inc) and stored at 4 °C until use.

2.3. Phage DNA sequencing

Genomic DNA of phages ALQ13.2 and ϕAbc2 was isolated using a QIAGEN Lambda Maxi kit with the modifications of Deveau et al. (2002). To sequence the genome of phage ALQ13.2, primers previously designed to sequence the genome of the *pac*-type phages 2972 (Lévesque et al., 2005) and 858 (Deveau et al., 2008) were used for direct sequencing of the conserved regions. New primers were then designed, as necessary, to complete the sequencing of both strands. All sequencing was carried out using an ABI Prism 3100 Genetic Analyzer at the Centre Hospitalier de l'Université Laval. The genome of phage ϕAbc2 was sequenced in a similar way except that primers previously designed to sequence the genome of the *cos*-type phage DT1 (Tremblay and Moineau, 1999) were used to obtain the first sequencing data. The *cos*-site was determined by comparison of the sequences obtained with linear and ligated phage DNA (Tremblay and Moineau, 1999). DNA sequence analysis, contig assembly, and editing were carried out using PreGap and Gap4 from Staden Package (http://staden.sourceforge.net/) (Staden et al., 2003) and BioEdit Sequence Alignment Editor (Hall, 1999). Open Reading Frame Finder graphical analysis tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html)

Table 1
Host range of *S. thermophilus* phages ALQ13.2 and Abc2 with some *S. thermophilus* strains.

Strain	Phage	
	ALQ13.2	ϕAbc2
ST13.2	+[a]	−[a]
Abc2	−	+
SMQ-301 [b]	−	−
RD534 [c]	−	−
4-C	−[a]	−[a]
5-C	−[a]	−[a]
15-C	−[a]	+[a]
M1-C	−[a]	−[a]
M10-C	−[a]	+[a]
M11-C	−[a]	−[a]
Sth10	−[a]	−[a]
799	−[a]	−[a]
ST3.1	−[a]	−[a]
ST10.3	−[a]	+[a]
CNRZ1066	−[a]	−[a]
LMD-9	−[a]	+[a]
LMG18311	−[a]	−[a]

[a] From Binetti et al., 2005.
[b] Host strain for phage DT1 (Tremblay and Moineau, 1999).
[c] Host strain for phage 2972 (Lévesque et al., 2005).

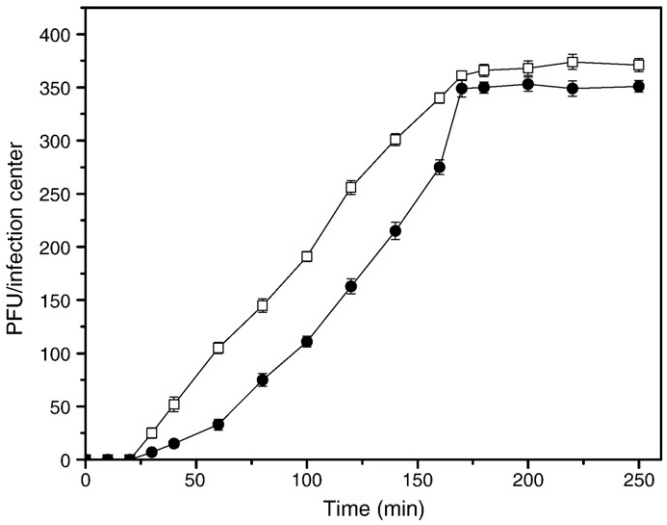

Fig. 1. One-step growth curves for *S. thermophilus* phages ALQ13.2 (□) and ϕAbc2 (●), using the corresponding sensitive strains ST13.2 and Abc2. The values are the means of three determinations.

and Heuristic GenMark (http://exon.gatech.edu/GeneMark/heuristic_hmm2.cgi) were used to define potential coding regions (Besemer and Borodovsky, 1999). Protein sequence comparisons were performed using BLASTP 2.2.18 from NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul et al., 1997).

2.4. Phages ALQ13.2 and ϕAbc2 structural protein analysis

We analyzed CsCl-purified phage particles for structural protein composition using SDS-PAGE with a 12% polyacrylamide separating gel and 4% polyacrylamide stacking gel (Sambrook and Russell, 2001). Phage samples were mixed with 2× sample loading buffer, boiled for 5 min and sonicated before loading. Protein bands were visualized using Coomassie blue stain. The protein bands of interest were excised from the gel, and identified by liquid-chromatography tandem mass spectrometry (LC-MS/MS) at the Centre Protéomique de l'Est du Québec (Université Laval, CHUQ-CHUL,). These results were analyzed using Scaffold Proteome Software (Craig and Beavis, 2003; Keller et al., 2002; Nesvizhskii et al., 2003).

2.5. Nucleotide sequence accession numbers

The nucleotide sequence of phages ALQ13.2 and ϕAbc2 genomes has been deposited in GenBank under accession no. FJ226752 and FJ236310, respectively.

3. Results and discussion

3.1. Host range

According to our results and previous host range studies (Binetti et al., 2005), phages ALQ13.2 and ϕAbc2 showed different host range profiles (Table 1). While ϕAbc2 revealed a broad range, infecting five different *S. thermophilus* strains (Abc2, 15-C, M10-C, ST10.3 and LMD-9), phage ALQ13.2 was able to infect only its host strain (ST13.2).

Table 2
Open reading frames deduced from the genome of *S. thermophilus* phage ALQ13.2 and their predicted functions.

ORF	Start	Stop	Size[a] (aa)	MW (KDa)	pI	Putative ribosome binding site and start codon (AAAGGAGGTGA)[b]	Best Matches (% amino acid identity)	Size[c] (aa)	Putative function
1	108	560	150 aa	16.82	6.05	TTTGCGGATGAatat **CTG**	ORF3 phage 858 145/150 (96%)	150	Terminase small subunit
2	547	1782	411 aa	47.07	5.65	AAAGGAGCTGTaagcg **ATG**	ORF26 phage O1205 391/411 (95%)	411	Terminase large subunit
3	1788	3296	502 aa	57.63	4.97	TAATTAGGAGAa **ATG**	ORF6 phage 858 479/502 (95%)	502	Portal protein
4	3293	4186	297 aa	34.26	9.30	AGGGTATATGA **ATG**	ORF6 phage 2972 282/297 (94%)	297	Capsid protein
5	4370	4951	193 aa	21.19	4.93	TAGATAGGAGAacata **ATG**	ORF7 phage 2972 173/193 (89%)	193	Scaffold protein
6	4973	5332	119 aa	12.72	8.75	AAAGGAATCTTaa **ATG**	ORF8 phage 2972 111/119 (93%)	119	Capsid protein
7	5350	6396	348 aa	37.66	4.89	AAAGGAGGAATattaaaac **ATG**	ORF9 phage 2972 337/348 (96%)	348	Major capsid protein
8	6408	6569	53 aa	6.03	7.93	AGAGGTACTGAt **ATG**	ORF10 phage 2972 49/53 (92%)	53	Capsid protein
9	6581	6922	113 aa	13.25	4.76	TAGTGAGGTATagc **ATG**	ORF11 phage 2972 105/113 (92%)	113	–
10	6919	7233	104 aa	11.59	9.58	AAAGAGGGAGAggtgctatttct **ATG**	ORF12 phage 2972 98/104 (94%)	104	–
11	7235	7579	114 aa	12.78	9.30	CAAGGTGGTGAaataac **ATG**	ORF114 phage Sfi11 103/114 (90%)	114	–
12	7576	7962	128 aa	14.73	5.38	AAATTGGATGAaac **ATG**	ORF14 phage 2972 118/128 (92%)	128	–
13	7976	8485	169 aa	18.59	4.89	TTAGGAGGAAAaa **ATG**	ORF15 phage 2972 162/169 (95%)	169	Major tail protein
14	8563	8916	117 aa	13.22	4.81	ATAGGAGTAAAcaaaca **ATG**	ORF17 phage 858 114/117 (97%)	117	–
15	8979	9296	105 aa	12.61	9.99	ATACGAGGAATtaatcactaatgct **ATG**	ORF17 phage 2972 101/105 (96%)	105	Tail protein
16	9320	13,924	1534 aa	155.59	9.56	AAAACATATAAccgtccgagcaata **TTG**	ORF18 phage 2972 1339/1534 (87%)	1517	Tail protein
17	13,924	15,459	511 aa	57.78	5.52	AGAGGTATTAAata **ATG**	ORF20 phage 858 487/511 (95%)	511	Tail protein
18	15,459	18,965	1168 aa	129.59	5.25	AAATTTGAGGAgagatatctata **ATG**	ORF21 phage 858 630/721 (87%)	1617	Receptor-binding protein
19	18,965	20,980	671 aa	74.56	6.29	GTAGGAGGTGCata **ATG**	ORF670 phage Sfi21 552/671 (82%)	670	Minor structural protein
20	20,996	21,406	136 aa	15.66	4.78	AAAGGAGATATaaaac **ATG**	ORF21 phage DT1 116/136 (85%)	131	–
21	21,426	21,572	48 aa	5.57	9.16	AAAGGATAAAAagat **ATG**	ORF22 phage DT1 48/48 (100%)	48	–
22	21,590	21,913	107 aa	12.52	5.79	ATAGGAGGGATgtgtt **ATG**	ORF23 phage DT1 95/107 (88%)	107	–
23	21,921	22,163	80 aa	8.85	8.09	AGAGGATGAAGaataa **ATG**	ORF24 phage DT1 78/80 (97%)	80	Putative holin
24	22,165	22,767	200 aa	21.92	4.67	AAAGGAGAAATaaa **ATG**	ORF25 phage DT1 195/200 (97%)	200	Putative lysin
25	22,974	23,735	253 aa	30.00	9.78	AAAGATGGTGTcataag **ATG**	ORF30 phage 858 244/253 (96%)	253	Endonuclease
26	23,798	24,025	75 aa	8.64	4.00	AAATGGAATCCctgtagca **ATG**	ORF27 phage DT1 69/75 (92%)	75	Lysin
27	24,764	25,315	183 aa	21.51	7.63	GAGAGAGGTAAacaaa **ATG**	ORF28 phage DT1 164/183 (89%)	183	–
28	25,502	25,320	60 aa	6.86	8.56	AAAGTTTATTTctgggaacata **TTG**	ORF127 phage Sfi21 33/36 (91%)	127	cI-like repressor
29	25,629	25,832	67 aa	7.71	8.01	AAAGGAGATAAcct **ATG**	ORF75 phage Sfi21 59/67 (88%)	75	Cro-like repressor
30	25,873	26,268	131 aa	15.40	6.16	AAAGGAACTAG **ATG**	*S. agalactiae* 515 59/94 (62%)	169	–
31	26,391	26,600	69 aa	8.23	8.86	AAAGGAGAAACga **ATG**	ORF31 phage 2972 54/69 (78%)	69	Cro-like repressor
32	26,634	26,774	46 aa	5.47	9.90	TATAGAGAGGAaccaaaa **ATG**	ORF30 phage DT1 42/46 (91%)	46	–
33	27,046	27,519	157 aa	17.98	6.20	GTAGGAATTAAat **ATG**	ORF33 phage 2972 152/157 (96%)	157	–
34	27,516	28,217	233 aa	26.47	6.34	AAAGGAGAAACcttaacatcag **ATG**	ORF9 phage O1205 227/233 (97%)	233	Nucleoside triphosphate binding motifs
35	28,192	29,523	443 aa	50.43	8.28	AAATTTGGTGAtttag **ATG**	ORF33 phage DT1 417/443 (94%)	443	Helicase
36	29,530	29,985	151 aa	17.27	4.89	TATGGAGATAAaaaact **ATG**	ORF36 phage 2972 150/151 (99%)	151	–
37	29,988	30,803	271 aa	30.48	7.07	AATTGACCTTCcgttctaatt **ATG**	ORF35 phage DT1 269/271 (99%)	271	–
38	30,784	32,304	506 aa	59.12	6.72	CAAAGAGATAAggagga **TTG**	ORF506 phage Sfi19 498/506 (98%)	506	Primase
39	32,340	32,155	61 aa	7.36	9.62	AAAGCAACCGAaaccctttattttca **ATG**	ORF61 phage Sfi21 56/59 (94%)	61	–
40	32,574	33,152	192 aa	22.92	5.37	TTAGGAGAAAAaatacttagagattaa **ATG**	ORF192 phage Sfi19 190/192 (98%)	192	–
41	33,168	33,653	161 aa	18.37	5.29	AATAGACTTTAt **CTG**	ORF166 phage Sfi18 160/161 (99%)	166	DNA binding protein
42	33,625	33,924	99 aa	11.08	9.37	AAAGGAGTAATga **TTG**	ORF98 phage Sfi19 89/98 (90%)	98	–
43	33,928	34,635	235 aa	27.97	9.20	TGAGGAGTTATtaagac **ATG**	ORF18 phage 7201 202/235 (85%)	235	–
44	35,071	35,469	132 aa	15.67	8.81	AAAGGAAAGACaattt **ATG**	ORF45 phage DT1 120/132 (90%)	132	–

[a] Number of amino acids (aa) of the predicted protein.
[b] Underlined, boldfaced letters indicate nucleotides identical to the RBS consensus; lowercase letters indicate spacer nucleotides between the RBS and start codon; boldfaced letters indicate the start codon.
[c] Number of amino acids (aa) of the best-matched protein.

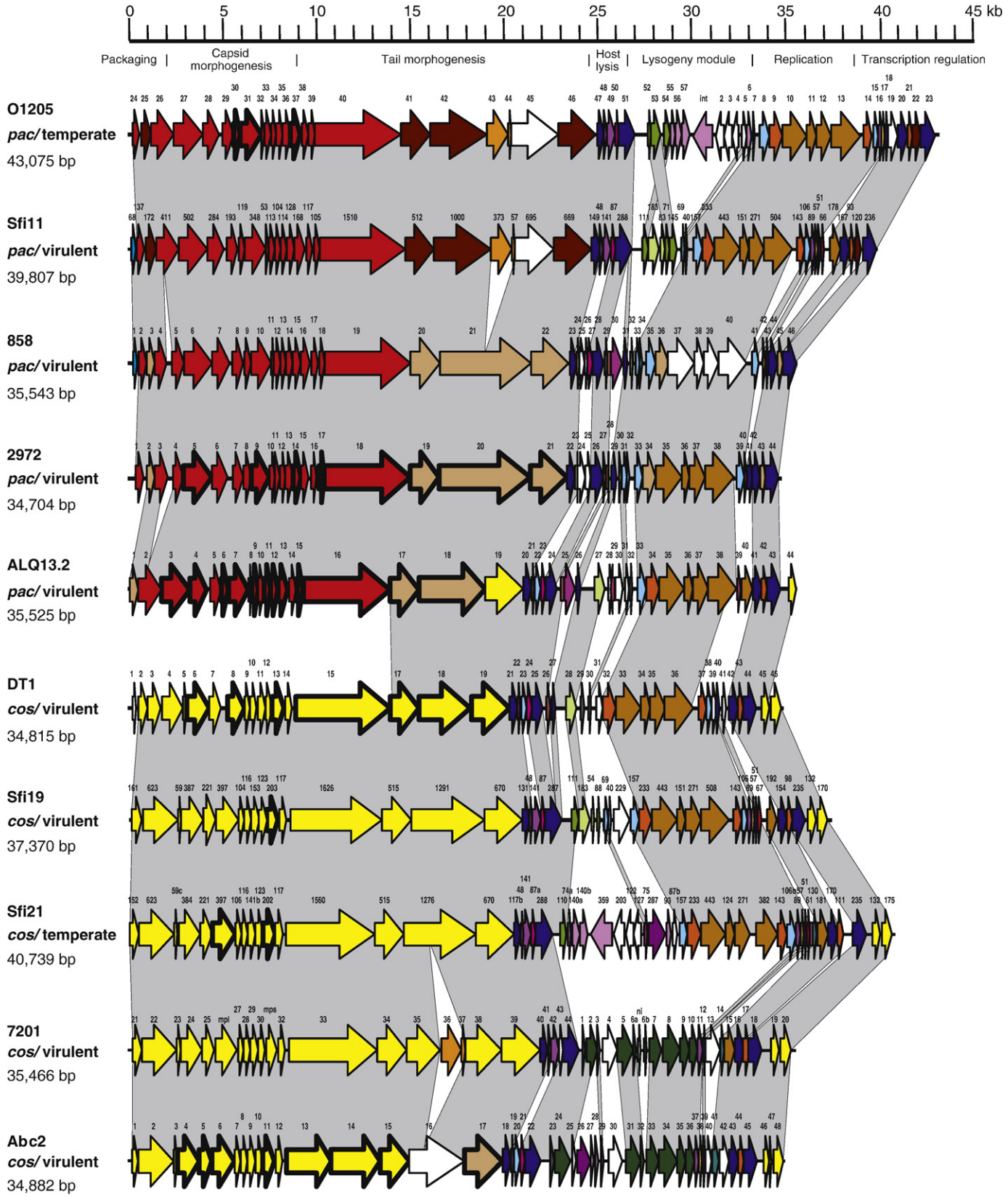

Fig. 2. Schematic representation of *S. thermophilus* bacteriophage genomes. ORFs connected by a grey box show homologies at the amino acid level; ORFs of the same color share more than 70% identity. ORFs with unique sequences are displayed in white. Genes coding for proteins identified by N-terminal sequencing, MALDI-TOF or LC-MS are identified by thick lines.

3.2. Burst size

Similarly to previous works (Suárez et al., 2002) *S. thermophilus* phages ALQ13.2 and ϕAbc2 showed rather large burst sizes (370 min and 350 min, respectively), with latent periods between 30 and 40 min, and burst times of 165 and 170 min, respectively (Fig. 1).

3.3. Genome of phage ALQ13.2

Phage ALQ13.2 genome was determined to be a 35,525 bp linear double-stranded DNA, similar to the *pac*-type virulent phage 858 genome size (35,543 bp, Deveau et al., 2008). Among *pac*-type phages, ALQ13.2 was found to be the second shortest genome after phage 2972 (34,704 bp, Lévesque et al., 2005). Its 39.4% G + C content was also similar to other *S. thermophilus* phage and host genomes (Desiere et al., 1999; Deveau et al., 2008; Le Marrec et al., 1997; Lévesque et al., 2005; Lucchini et al., 1998; Stanley et al., 1997; Tremblay and Moineau, 1999). Sequence analysis revealed 44 ORFs containing 40 or more codons (Table 2). Each *orf* was preceded by a region sharing variable identities with the Shine–Dalgarno sequence complementary to the 3′ end of the 16S rRNA of *S. thermophilus* (AAAGGAGGTGA). Of the 44 ORFs, 24 were assigned a putative function based on their similarities to proteins with known functions or conserved motifs. The highest similarities (between 78% and 99%) were observed with ORFs from the phage 2972 genome (Lévesque et al., 2005) (Figs. 2 and 3). A total of 14 ORFs (32%) from phage ALQ13.2 had their best match with phage 2972 ORFs, including several structural proteins. Despite considerable homology with the virulent *pac*-type phage 2972, significant similarities were also found with the virulent *cos*-phage DT1, since 11 ORFs (ORFs 20 to 24, 26, 27, 32, 35, 37, and 44) demonstrated the best match with proteins from this phage, including the two proteins of the lysis system, the holin and the endolysin (Table 3, Figs. 2 and 3). Of note, ORF30 of phage ALQ13.2 did not match a phage protein but shared 62% similarity with a protein of unknown function from *Streptococcus agalactiae* strain 515 (GenBank accession no. ZP_00790470).

3.4. Genome of phage ϕAbc2

Its genome was the second shortest (34,882 bp) among *cos*-type *S. thermophilus* phages, falling between DT1 (34,820 bp, Tremblay and Moineau, 1999) and 7201 in size (35,466 bp, Le Marrec et al., 1997). Phage ϕAbc2 contained a linear double-stranded DNA genome with 39.0% G + C content. We identified 48 *orf*s, 26 of which coded for proteins with known functions (Table 3). The *cos*-site had a 5′ overhang, was 20 nucleotides long (5′-CCACGACAAGGTGTCTTCTC-3′), and was

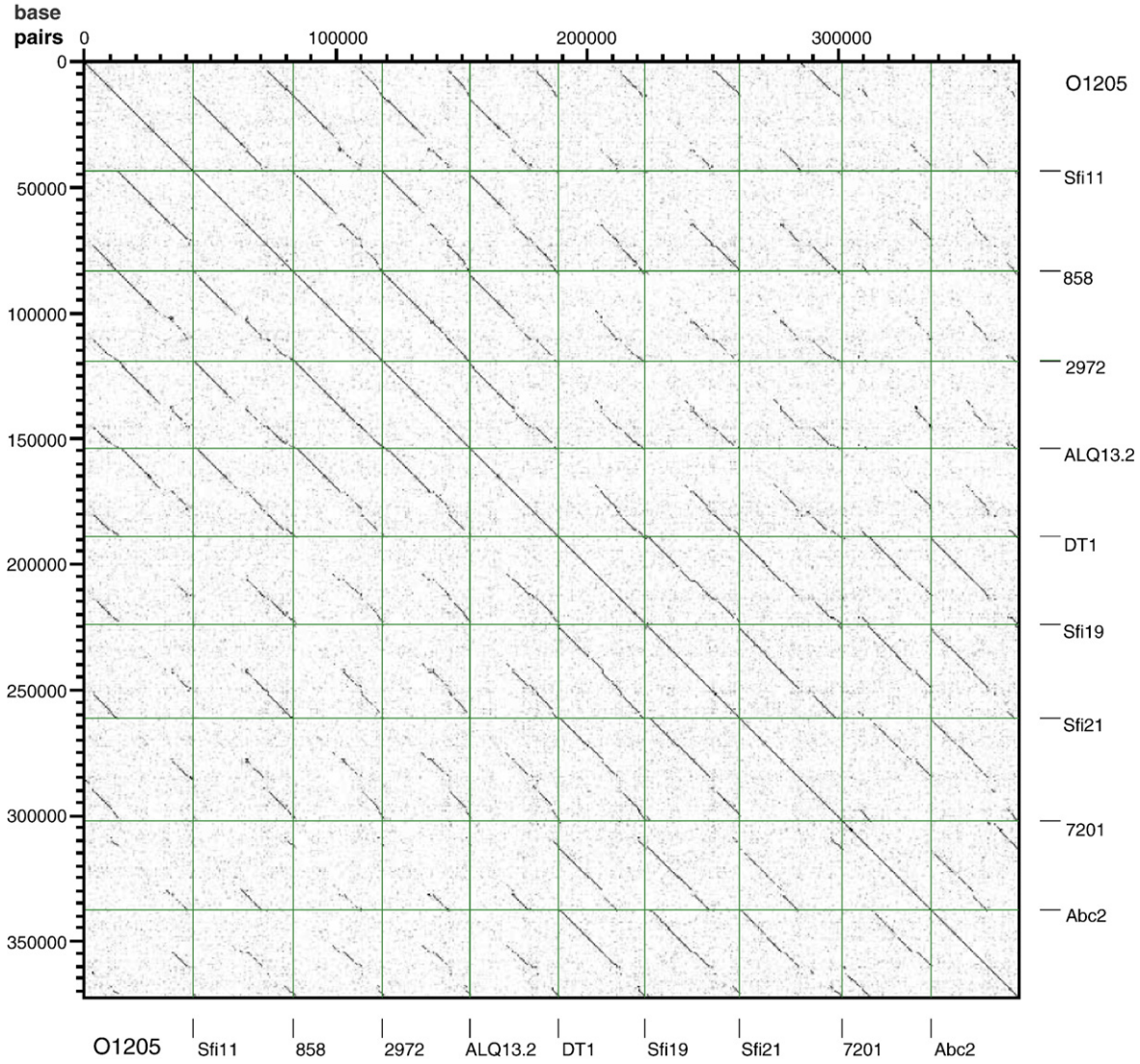

Fig. 3. Dotplot matrix comparison calculated for the complete nucleotide sequences of *S. thermophilus* phages O1205, ϕSfi11, 858, 2972, ALQ13.2, DT1, ϕSfi19, ϕSfi21, 7201 and ϕAbc2. The dotplot matrix was calculated using Dotter (Sonhammer and Durbin, 1995).

very similar to those identified for phages DT1 (5′-CCACCACAAGGTG-3′) (Tremblay and Moineau, 1999), ϕSfi19 (5′-CCACGACAAGGTGTT-3′) (Lucchini et al., 1999a), and ϕSfi21 (5′-CCGCCACAAGGTGTC-3′) (Desiere et al., 1999).

High homology with phage DT1 was observed since the best matches for 27 ϕAbc2 ORFs (56%) were with proteins from DT1, including capsid and tail proteins (Figs. 2 and 3). Surprisingly, ϕAbc2 contained 6 ORFs (31 to 36) highly similar (86–95%) to deduced proteins possibly involved in the replication of phage 7201 (ORF5 to ORF10) (Lévesque et al., 2005). Up to date, phage ϕAbc2 is the second *S. thermophilus* phage known to contain this unusual replication module. Of interest, we found significant similarity (98%) between ORF28 of the *cos*-type phage ϕAbc2 and a protein of unknown function in the temperate *pac*-type *S. thermophilus* phage O1205. Likewise, ORF17 of phage ϕAbc2 shared 85% similarity with a tail protein found in the *pac*-type *S. thermophilus* phage 2972 (Lévesque et al., 2005). Not all of the deduced ϕAbc2 proteins had homologs in *S. thermophilus* phage genomes. For example, ORF25 and ORF30 had 64% and 50% similarity, respectively, to proteins from two different *Streptococcus pyogenes* phages, while ORF40 had 60% similarity with a putative *Streptococcus pneumoniae* prophage MM1 protein. In some cases the ORFs did not match with a phage protein: ORF27 showed some similarities (36%) with a protein of unidentified function from *S. agalactiae* strain 2603 V/R (GenBank accession no. NP_687585). Thus, an ancestor of phage ϕAbc2 appears to have acquired some genes from other streptococcal phages and be amongst the most divergent (Fig. 2).

3.5. Genome organization

Like the other *S. thermophilus* phages, the genomes of the two Argentinean phages are organized into distinct modular regions (Desiere et al., 2002; Lévesque et al., 2005). The schematic representation of the ORFs from the ten complete sequenced *S. thermophilus* phages (Fig. 2) and the dotplot analysis show comparison between

Table 3
Open reading frames deduced from the genome of *S. thermophilus* phage ϕAbc2 and their predicted functions.

ORF	Start	Stop	Size[a] (aa)	MW (KDa)	pI	Putative ribosome binding site and start codon (AAAGGAGGTGA)[b]	Best Matches (% amino acid identity)	Size[c] (aa)	Putative function
1	234	545	103	11.3	4.7	C**AACG**CAT**TGA**tacgttc TTG	ORF2 phage DT1 99/103 (96%)	153	Terminase small subunit
2	567	2438	623	71.5	4.7	**AAAGGAG**CAACa GTG	ORF623 phage Sfi19 594/623 (95%)	623	Terminase large subunit
3	2442	2621	59	6.6	8.1	A**GAGGAG**TATTaatat ATG	ORF5 phage DT1 57/59 (96%)	59	Head–tail joining protein
4	2639	3799	386	42.8	5.1	**AAAGGAGGTGA**taacaa ATG	ORF6 phage DT1 370/386 (95%)	386	Portal protein
5	3786	4454	222	24.5	4.9	**AAAGGAGGTGA**gataa ATG	ORF7 phage DT1 221/222 (99%)	222	Scaffolding protein
6	4469	5662	397	44.2	5.1	**AAAGGA**AAATAatta ATG	ORF397 phage Sfi19 370/397 (93%)	397	Major capsid protein
7	5677	5991	104	11.5	4.2	TT**AGGAGGT**AAgct ATG	ORF9 phage DT1 104/104 (100%)	104	DNA packaging protein
8	5991	6341	116	13.4	9.6	G**AAAGAGGTGA**cta ATG	ORF10 phage DT1 115/116 (99%)	116	Capsid–tail joining protein
9	6348	6770	140	15.6	9.3	**AA**GTTG**GGTGA**tagctt ATG	ORF11 phage DT1 140/140(100%)	140	Tail component protein
10	6775	7146	123	14.1	4.4	**AAGGAGG**G**GA**gtaattaa GTG	ORF12 phage DT1 122/123 (99%)	123	Tail component protein
11	7168	7779	203	21.9	5.7	**AAAGGAG**AAAAtatat ATG	ORF13 phage DT1 200/203 (98%)	203	Major tail protein
12	7854	8207	117	13.5	4.6	**AAAGGAG**TAA**A**gacaca ATG	ORF14 phage DT1 117/117 (100%)	117	Tail component protein
13	8426	10,816	796	87.6	9.3	**AAAGGAGG**GAAtataac ATG	ORF15 phage DT1 781/795(98%)	1656	Tail component protein
14	10,779	13,397	872	96.4	9.3	**AAA**AAATT**TG**Ttg ATG	ORF15 phage DT1 848/865(98%)	1656	Tail component protein
15	13,394	14,953	519	58.4	5.6	TT**AGGAGGT**CAaattat TTG	ORF17 phage DT1 513/519(98%)	518	Tail component protein
16	14,932	17,865	977	109.9	5.2	CC**A**ACA**A**T**TGA**aatttc ATG	ORF18 Phage DT1.1 639/975 (65%)	905	Host specificity protein
17	17,866	19,887	673	73.8	7.6	GT**AGGAGGT**TTttaa TTG	ORF21 phage 2972 576/673 (85%)	673	Tail protein
18	19,903	20,313	136	15.6	4.7	**AAAGGAG**ATATaaaac ATG	ORF21 phage DT1 114/136(83%)	131	–
19	20,333	20,479	48	5.6	9.4	**AAAGGA**TA**A**A**A**agat ATG	ORF22 phage DT1 47/48 (97%)	48	–
20	20,497	20,820	107	12.4	6.0	G**AGGGA**T**GTG**Tt ATG	ORF23 phage DT1 94/107 (87%)	107	–
21	20,828	21,070	80	8.8	6.5	**AGAGGA**TAATAataaa ATG	ORF24 phage DT1 72/80 (90%)	80	Holin
22	21,072	21,917	281	31.2	4.3	**AAAGGAG**AAATaaa ATG	ORF44 phage 7201 247/281(87%)	281	Lysin
23	22,417	22,620	67	7.6	9.2	G**AAGGAGG**AACaaa ATG	ORF29 phage DT1 63/67 (94%)	67	Cro-like protein homolog
24	22,661	23,488	275	31.6	5.3	**AAAGGA**ACAAT ATG	ORF2 phage 7201 137/172 (79%)	175	–
25	23,587	23,790	67	7.6	5.0	G**AAGGAGG**ACAcaa ATG	*S. pyogenes* str. Manfredo phage 43/67 (64%)	75	–
26	23,845	24,561	238	27.0	9.5	**AAA**T**GAG**AGA**T**acga ATG	*Streptococcus* phage TP-J34 233/238 (97%)	238	Phage antirepressor protein
27	24,571	24,720	49	5.6	9.7	TT**AGGAGG**GC**A**ac ATG	*S. agalactiae* 2603 V/R 16/44 (36%)	47	–
28	24,804	24,974	56	6.6	9.1	C**A**CT**GA**TT**TA**a CTG	ORF6 phage O1205 51/52 (98%)	93	–
29	25,114	25,257	47	5.4	9.7	**AAAGGA**A**T**TT**A**aaaa ATG	ORF30 phage DT1 41/47 (87%)	46	–
30	25,514	26,245	243	28.8	6.6	GG**AG**AG**GGTGA**gtctaaa ATG	*S. pyogenes* phage MGAS8232 90/179 (50%)	312	–
31	26,433	27,215	260	30.3	8.2	CTA**AGAGGT**TCtttat ATG	ORF5 phage 7201 234/260 (90%)	260	DnaC homolog
32	27,212	27,394	60	7.3	6.2	C**AAG**AG**GA**T**GA**tgtt ATG	ORF6 phage 7201 53/60 (88%)	60	–
33	27,522	28,178	218	25.0	6.2	A**GAGGA**TA**TGA**c ATG	ORF7 phage 7201 208/218 (95%)	218	Erf protein
34	28,181	29,152	323	37.6	5.0	**AACGGA**A**GGA**taaat ATG	ORF8 phage 7201 291/318 (91%)	319	–
35	29,149	29,601	150	16.8	5.8	**AAAGGAG**AAAAcaa ATG	ORF9 phage 7201 129/150 (86%)	148	Single-stranded binding protein
36	29,611	30,072	153	18.0	9.4	GTCTA**AGGTGA**act ATG	ORF10 phage 7201 135/153 (88%)	153	–
37	30,069	30,305	78	9.1	9.0	C**AAGGAGTTG**Ga ATG	ORF40 phage 2972 64/78 (82%)	83	–
38	30,296	30,469	57	6.4	6.2	G**AAAGAGA**T**GA**tagaact ATG	ORF57 phage Sfi19 51/57 (89%)	57	–
39	30,507	30,665	52	6.4	5.2	GGC**GGAGGAAA**tataaa ATG	ORF41 phage DT1 41/51 (80%)	51	–
40	30,662	30,979	105	12.1	9.6	TG**AGGAGGT**A**G**atga ATG	*S. pneumoniae* phage MM1 12/20 (60%)	165	–
41	31,008	31,391	127	14.3	7.7	**ATTGGAG**AAATtaa ATG	ORF130 phage Sfi21 108/127 (85%)	130	–
42	31,403	31,903	166	19.8	4.4	T**AAGGAG**AACTaga ATG	ORF178 phage Sfi11 112/173 (64%)	178	–
43	31,904	32,410	168	19.2	5.3	G**AAG**AG**GTTGA**ataa GTG	ORF42 phage DT1 163/168 (97%)	165	DNA binding protein
44	32,385	32,687	100	11.0	9.1	**AAAGGA**ATAATgattg ATG	ORF43 phage DT1 96/100 (96%)	100	–
45	32,684	33,391	235	27.7	9.3	**AAAGGA**A**GA**G**G**gca ATG	ORF44 phage DT1 220/235 (93%)	235	–
46	33,738	34,037	99	11.6	9.2	**AAA**TAATC**TGA**caacattattatacc ATG	ORF45 phage DT1 92/92 (100%)	132	–
47	34,030	34,158	42	5.0	8.9	T**ATTGAGG**ATA**A**tag ATG	ORF45 phage DT1 40/40 (100%)	132	–
48	34,270	34,788	172	19.9	9.6	**AGAGGAGG**GA**A**gcca ATG	ORF46 phage DT1 172/172 (100%)	185	HNH endonuclease

[a] Number of amino acids (aa) of the predicted protein.
[b] Underlined, boldfaced letters indicate nucleotides identical to the RBS consensus; lowercase letters indicate spacer nucleotides between the RBS and start codon; boldfaced letters indicate the start codon.
[c] Number of amino acids (aa) of the best-matched protein.

them (Fig. 3). Though these isolates were geographically remote from the other sequenced *S. thermophilus* phages, the genome organizations were remarkably identical.

Some genomic regions are also highly conserved in all *S. thermophilus* phage genomes, especially those involved in DNA replication and host cell lysis (Lévesque et al., 2005). In addition, genes involved in transcription regulation are also well-conserved. However, there are some exceptions such as the genes involved in the replication of phages 7201 and ϕAbc2 which are similar to each other but dissimilar from the 8 other *S. thermophilus* phages. The region comprising *orf*37 to *orf*40 is still unique to phage 858 (Deveau et al., 2008). Within those conserved regions, some genes are highly conserved in all 10 phages. Such genes include the *orf*24 (of phage ALQ13.2) coding for the endolysin as well as *orf*20 and *orf*21 for which no putative function could be assigned. Similarly, inside the transcription regulation module, *orf*41 (coding for a putative DNA binding protein) and *orf*43 (possibly a transcription regulation gene) are also highly conserved in the 10 *S. thermophilus* phages. Such conserved genes suggest a key role in the phage lytic cycle and perhaps could be used to design specific primers for rapid detection of *S. thermophilus* phages (Binetti et al., 2005; del Rio et al., 2007, 2008; Quiberoni et al., 2006).

The genes coding for proteins involved in the morphogenesis are highly conserved but only within each of the *pac*- and *cos*-groups. Interestingly, some diversity was observed in the deduced tail and baseplate proteins within the *pac*-type phages. Three subgroups could be observed, namely ϕO1205/ϕSfi11, 858/2972 and ALQ13.2. The latter Argentinean phage is rather interesting as one of its tail proteins is more related to *cos*-type phages. Within the *cos*-type phages, the tail proteins are more similar. Of note, ORF17 from *cos*-phage ϕAbc2 shows the highest similarities (85%) with ORF21 of the *pac*-type phage 2972 (Lévesque et al., 2005). Clearly, these two groups of *S. thermophilus* phages appear to have exchanged some genes.

3.6. Phage origin of replication

Phage origins of replication (*ori*) are mainly characterized by a non-coding region containing several inverted and direct repeats (Hill et al., 1990). In all *S. thermophilus* phage genomes analyzed to date, the *ori* was located just upstream of the genes coding for proteins involved in DNA replication (Lévesque et al., 2005). For phage ALQ13.2, the putative origin of replication was identified within a non-coding 417 bp region located between *orf*39 and *orf*40. The region included several inverted and direct repeats and had an A + T content of 67.5%. Previous studies identified two different origins of replication for phage ϕ7201 (Stanley et al., 2000). They were designated *ori*7201A and *ori*7201B and were located within *orf*4, *orf*5, *orf*6 and *orf*7. Notably, a region of phage ϕAbc2 closely matched (83% similarity) the sequence corresponding to *ori*7201B, which consisted of a 580-bp fragment spanning part of *orf*31, all of *orf*32 and part of *orf*33. An anti-phage system named PER (phage encoded resistance) was previously associated with the presence of the phage *ori* provided on a plasmid (Hill et al., 1990). It is presumed that phage replication factors are titrated by the plasmid harboring the phage *ori*. This effect was also observed in *S. thermophilus* with the *ori* from phages ϕSfi19, ϕSfi21, ϕO1205, 7201, and DT1 (Foley et al., 1998; Lamothe et al., 2005; Stanley et al., 2000; Sturino and Klaenhammer, 2002).

3.7. Lysogeny modules

The genomes of temperate bacteriophages ϕO1205 (Stanley et al., 1997) and ϕSfi21 (Desiere et al., 1999) comprise the lysogeny module, found between the lysis cassette and the replication module (Fig. 2). Some virulent phages, such as 2972, DT1, ϕSfi11 and ϕSfi19, contain fragments of the lysogeny module (Lévesque et al., 2005). Likewise, phages ϕAbc2 and ALQ13.2 have one (*orf*23) and three *orf*s (*orf*28, *orf*29 and *orf*31), respectively, coding probably for cI or *cro*-like repressors usually found in lysogeny modules (Tables 2 and 3). The presence of these genes suggests that ALQ13.2 and ϕAbc2 may have arisen from temperate phages following a deletion in the lysogeny module as previously observed for phage ϕSfi21 (Bruttin and Brüssow, 1996). It has been also suggested previously that the lysogeny module appears to be a recombination hot spot in *S. thermophilus* phages (Lucchini et al., 1999b).

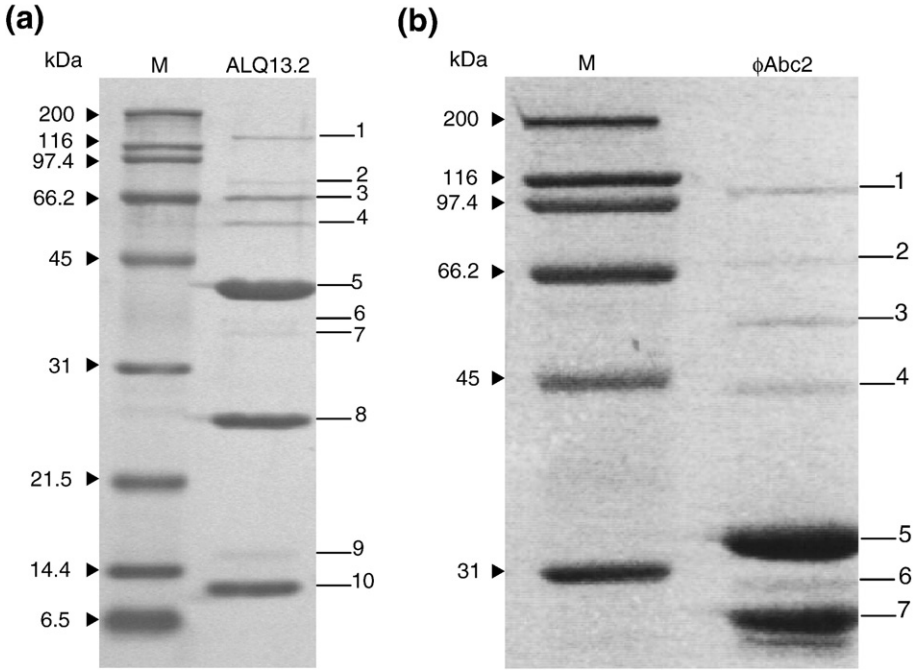

Fig. 4. Coomassie blue-stained 12% SDS-Polyacrylamide gel showing the structural proteins of *S. thermophilus* phages ALQ13.2 (a) and ϕAbc2 (b). M: molecular mass standard. Values shown on left. Numbers to the right represent protein bands that were excised and subsequently analyzed and identified using LC-MS/MS.

Table 4
Analysis of ALQ13.2 and ϕAbc2 structural proteins by LC-MS/MS.

Phage/ Band #	ORF	Molecular weight (MW) (kDa)		Putative function
		SDS-PAGE	Calculated	
ALQ13.2				
1	18	144	129	Antireceptor
2	16	81	155	Tail protein
3	3	66	58	Portal protein
4	17	61	58	Tail protein
5	7	41	38	Major capsid protein
6	6	38	13	Capsid protein
7	4	36	34	Minor capsid protein
8	13	27	19	Major tail protein
9	12	16	15	Unknown
10	15	11	13	Tail protein
	11		13	Unknown
	9		13	Unknown
ϕAbc2				
1	13	118.4	87.6	Tail protein
	17		73.8	Tail protein
2	15	71.0	58.5	Tail protein
3	4	55.6	42.8	Portal protein
4	14	45.3	96.4	Tail protein
5	6	43.0	44.0	Major capsid protein
6	5	30.2	24.5	Scaffolding protein
7	11	28.6	21.9	Major tail protein

3.8. Morphogenesis and structural proteome

Analysis of phage ALQ13.2 by SDS-PAGE revealed ten structural proteins (Fig. 4a, Table 4). Typical for a *pac*-type *S. thermophilus* phage (Le Marrec et al., 1997), three of the bands corresponded to major structural proteins of approximately 41 kDa, 25 kDa, and 13 kDa (Fig. 4a, protein bands #5, #8 and #10; ORF7, ORF13 and ORF15, respectively). Seven other minor structural proteins were also identified. Seven of the protein bands (#1, #2, #3, #4, #6, #7 and #9) contained a single protein associated with one phage deduced protein (ORF18, ORF16, ORF3, ORF17, ORF6, ORF4, and ORF12, respectively). One protein band (#10) contained three different phage proteins (ORF15, ORF11, and ORF12). Though the putative functions of two out of these three ORFs (9 and 11) have not yet been determined, the location of their genes suggests that they are related to capsid-morphogenesis proteins. The molecular mass estimated by SDS-PAGE for band #2 (ORF16) was 81 kDa, while the calculated mass was 155 kDa, suggesting that this protein is cleaved. The estimated molecular mass for band #6 (ORF6) was 38 kDa, but its calculated mass was 13 kDa, suggesting that this protein may form multimers.

Protein analysis of phage ϕAbc2 was carried out as for phage ALQ13.2, leading to the identification of seven protein bands (Fig. 4b, Table 4). Band #1 was found to contain two phage proteins (ORF13 and ORF17), while the other bands were each associated with a single protein. Bands #5 and #7 were related to major capsid and tail proteins (ORF6 and ORF11, respectively), while bands #2 and #4 were identified as putative tail proteins (ORF15 and ORF14, respectively). The molecular mass estimated by SDS-PAGE for protein band #4 was 45.3 kDa while the calculated mass was 96.4 kDa, suggesting dimer formation.

3.9. Phage classification

Greater *S. thermophilus* phage diversity has been postulated to exist in cheese plants compared to yogurt factories (Brüssow et al., 1994; Quiberoni et al., 2003, 2006). The genome sequence of phage ALQ13.2 is the first corresponding to a *pac*-type phage isolated from a cheese process. Given the high degree of similarity between phage ALQ13.2 and the *S. thermophilus* phages previously sequenced (at both the nucleotide and proteome levels), our results support the current *S. thermophilus* phage classification scheme based on type of DNA packaging mechanism (*cos* or *pac*) and structural protein composition, regardless of the isolation source.

4. Conclusions

Despite the distinctive features that have been reported for Argentinean *S. thermophilus* phages (Quiberoni et al., 2006; Suárez et al., 2002), analysis of the complete nucleotide sequence and the structural proteomes of phages ALQ13.2 and ϕAbc2 led us to conclude that they are closely related to previously sequenced *S. thermophilus* phages, even when the latter have been isolated geographically far from the former ones. Thus, our results suggest that *S. thermophilus* phage diversity is not extensive. This is strikingly different from phages from other lactic acid bacteria used in dairy starter cultures. For example, *Lactococcus lactis* phages are divided in at least 10 genetically distinct groups (Deveau et al., 2006), with various groups prevailing in different countries.

Comparative genomics and multilocus sequencing analyses previously suggested that the *S. thermophilus* species recently emerged and is still undergoing a process of regressive evolution towards a specialised bacterium for growth in milk (Hols et al., 2005). Moreover, *S. thermophilus* may have a clonal structure. Such limited diversity may explain why strains of this species are sensitive to similar phages. It has been also argued that *S. thermophilus* evolves mainly via recombination with other *S. thermophilus* strains (Rasmussen et al., 2008). It is very likely that a similar phenomenon is observed with their phages. Virulent phages can exchange new DNA from prophages or other virulent phages as it has been observed for other dairy phages (Bouchard and Moineau, 2000; Labrie and Moineau, 2007; Moineau et al., 1994). Genome analyses suggest that phages ALQ13.2 and ϕAbc2 are likely functional derivatives resulting from recombination events between *S. thermophilus* phages.

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