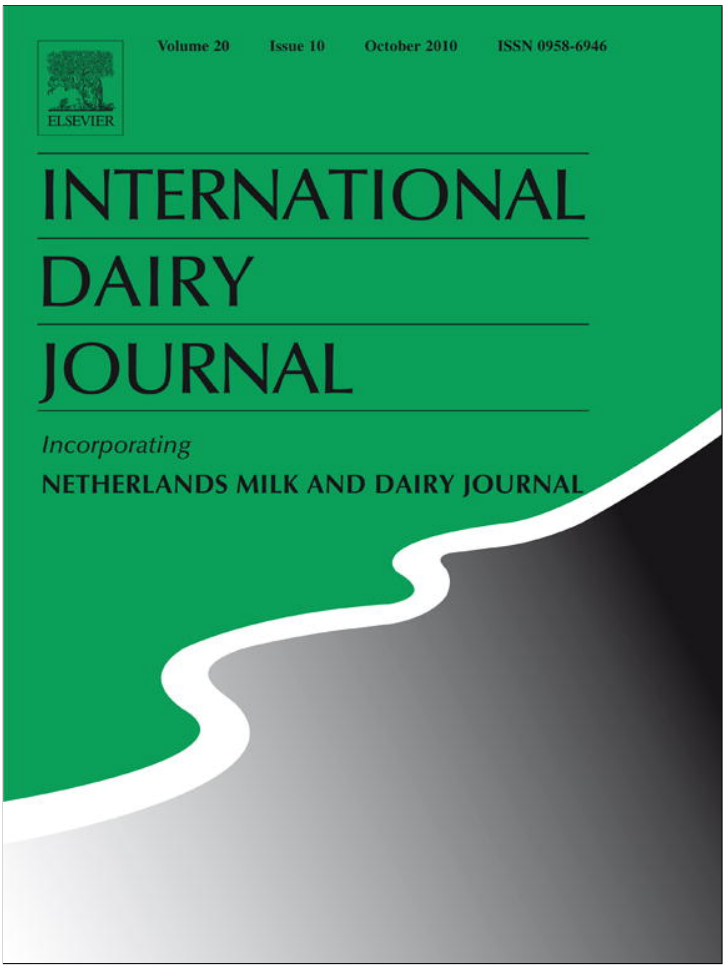

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Review

Streptococcus thermophilus bacteriophages

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ABSTRACT

At least 345 bacteriophages infecting *Streptococcus thermophilus* starter cultures have been isolated; general characteristics include high thermal resistance, short latent periods and large burst size. Phages with such characteristics are primed to thrive in the cheese making environment, lysing bacterial cultures and generating low-quality fermented products. All *S. thermophilus* phages isolated to date are members of the *Siphoviridae* family and the *Caudovirales* order and appear to constitute a polythetic phage species comprising two large groups, *cos*- and *pac*-types, based on the mode of DNA packaging. Comparative analyses have shown that *S. thermophilus* phage genomes are similarly organized into distinct modular regions and allow the detection of a core genome region. Several PCR-based techniques have been designed to detect them in cheese whey and milk samples. Similar *S. thermophilus* phages are globally distributed and endemic in specific dairy environments. The biogeography of *S. thermophilus* phages reinforces their current classification.

Contents

1. Introduction

Streptococcus thermophilus is one of the commercially most important lactic acid bacteria (LAB). This species is used, along with *Lactobacillus* strains, in starter cultures for the manufacture of several fermented dairy foods, including fermented milks, yogurt, and various Swiss and Italian cheeses (Boucher & Moineau, 2001; Delcour, Ferain, & Hols, 2000; Pearce & Flint, 2003). The inclusion of *S. thermophilus* to the pure mesophilic blends (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) allowed lower inoculation levels during Cheddar manufactures (Lucey, 2003). *S. thermophilus* strains are also largely used in starter cultures for several varieties of Argentinean soft and semi-hard cheeses (Cremoso, Cuartirolo, Holanda, Fontina, Colonia and Pategrás) (Reinheimer et al., 1997).

During the past two decades, a significant increase in the consumption of these milk products has greatly augmented the worldwide use of *S. thermophilus*. Products based on this bacterium have a now worldwide market value of approximately 40 billion dollars per year (Blomqvist, Steinmoen, & Håvarstein, 2006). Considering that approximately 10^{11} bacterial cells are needed to produce 1 kg of cheese, it is clear that market pressure has led to new demands on the performance of *S. thermophilus* cultures. On

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the other hand, the extensive use of LAB on an industrial scale has generated biotechnological problems such as phage infection (Moineau & Lévesque, 2005).

It is widely acknowledged that an increased productivity within existing facilities will lead to milk fermentation failures due to virulent phages (Brüssow, 2001; Sturino & Klaenhammer, 2006). For decades, the dairy industry has relied on an array of strategies to control this natural phenomenon (for a review, see Emond & Moineau, 2007). In spite of these extensive efforts, phage "attacks" remain today the most common cause of slow or incomplete milk fermentation (Sturino & Klaenhammer, 2006).

Phage attacks against mesophilic *Lactococcus lactis* strains have been reported more frequently in the literature but a similar phenomenon is now been observed with *S. thermophilus* strains as more thermophilic cultures are been used worldwide. During the last 55 years, numerous studies from Europe, Canada, the United States, and Argentina reported the isolation of several virulent bacteriophages for *S. thermophilus* (Table 1) and the identification of a few temperate phages. Although the literature includes large numbers of phages for *S. thermophilus*, their origin, geographical distribution, morphology, and general properties have not been extensively reviewed.

2. Geographical origin of phages and dairy products involved

Krusch, Neve, Luschel, and Teuber (1987) observed a marked host specificity in phages from France, Switzerland, and Germany, suggesting that these countries used specific thermophilic streptococci in yogurt and cheese production. Simultaneously, Brüssow, Bruttin, Desiere, Lucchini, and Foley (1998) postulated that *S. thermophilus* phages differed according to their geographical origin. The first studies reporting the isolation of *S. thermophilus* phages originated 55 years ago in Switzerland (Deane, Nelson, Ryser, & Carr, 1953; Kiuru & Tybeck, 1955). Since then, at least 345 virulent and temperate *S. thermophilus* phages have been described in many countries, mostly in North America and Europe. Over 110 *S. thermophilus* phages were also isolated and characterized in Argentina, thus in a country geographically very distant from these regions (Table 1).

The manufacture of many artisanal cheeses in several countries still depends on the use of undefined natural whey and milk cultures. However, large and modern industrial-scale cheese plants require starters that give more reproducible performance and uniform quality of the final fermented products (Powel, Broome, & Limsowtin, 2003). An unfortunate consequence of this has been the decrease of natural microbial diversity of many fermented dairy products. In *S. thermophilus*, this phenomenon is also reinforced in view of the fact that selecting an industrial strain for these defined cultures is a long and costly process because as many as 15 phenotypes must be characterized for each strain (Klaenhammer et al., 2002). This is an obvious incitation for their extensive use. Moreover, the quest for uniform and exclusive products favours the repeated use of key strains, leading to the appearance of an array of related virulent phages for the restricted number of *S. thermophilus* strains composing commercial starters worldwide.

S. thermophilus phages are now assembled into only two distinct groups according to their DNA packaging mechanism (*cos* or *pac*) and the numbers of major structural proteins. Specifically, Le Marrec et al. (1997) found a strict correlation between the presence of a particular set of major structural phage proteins and the mechanism of DNA packaging, demonstrating that *cos*-containing phages possessed two major structural proteins (32 and 26 kDa), in contrast to *pac*-containing phages which possessed three major structural proteins (41, 25, and 13 kDa).

Still, as shown by DNA restriction patterns and host range, a great diversity of phages is often observed in cheese making, (Brüssow et al., 1998; Quiberoni, Tremblay, Ackermann, Moineau, & Reinheimer, 2006; Quiberoni et al., 2003). In contrast, several authors from Europe and Argentina reported a more homogenous population of phages in yogurt production facilities (Benbadis, Faelen, Slos, Fazel, & Mercenier, 1990; Brüssow et al., 1994, 1998; Bruttin et al., 1997; Fayard, Haeflinger, & Accolas, 1993; Larbi, Colmin, Rousselle, Decaris, & Simonet, 1990; Neve, Krusch, & Teuber, 1989; Quiberoni et al., 2003; Suárez, Quiberoni, Binetti, & Reinheimer, 2002). The diversity of phage populations in cheese making may be due to the rotation of multiple strains of *S. thermophilus* in starter cultures, as compared to yogurt starters, which are much less varied (Brüssow & Kutter, 2005; Quiberoni et al., 2006). Indeed, a limited number of commercial *S. thermophilus* strains possess the required industrial phenotypes for yogurt manufacture, for example the capacity of exopolysaccharide production (Broadbent, McMahon, Welker, Oberg, & Moineau, 2003).

In recent years, several ecological studies were carried out to elucidate the origin of *S. thermophilus* phages in dairy environments (Brüssow & Desiere, 2001; Brüssow et al., 1998; Bruttin et al., 1997). Bruttin et al. (1997) identified raw milk as the source of new *S. thermophilus* phages infecting a mozzarella cheese factory, even if they were present in low titers ($<$130 pfu mL^{-1}). Their presence in dairy products is enhanced by their ability to survive the pasteurization of raw milk. High thermal resistance was exhibited by several *S. thermophilus* phages (Suárez et al., 2002) which remained infectious even after 45 min at 63 °C or 12 min at 72 °C. Lysogeny was long thought to be co-responsible for phage infections. Oddly, lysogenic *S. thermophilus* strains are rare in industrial strains as only 1–2.2% of these are induced by mitomycin C (Brüssow & Kutter, 2005; Carminati & Giraffa, 1992). These data contrast with a frequency of 25% inducible *S. thermophilus* strains found by Smaczny and Krämer (1984), or an incidence of 10% reported by Fayard et al. (1993). The role of lysogeny in the milk fermentation process is still unsettled, although prophages have been linked to the autolytic phenotype of some *S. thermophilus* strains (Husson-Kao et al. 2000) and the emergence of virulent phages (Bruttin, Foley, & Brüssow, 2002).

Data on growth parameters for *S. thermophilus* phages are scarce (Binetti, 2001; Deveau et al., 2008; Larbi et al., 1990; Tremblay & Moineau, 1999). Remarkably short latent periods (20–40 min) and very large burst sizes (ranging from 300 to 600 pfu per infection centre) were reported by Binetti (2001) for five of these phages. Phage DT1 has a burst size of 276 $\pm$ 36 and a latent period of 25 min. Deveau et al. (2008) reported a burst size of 190 $\pm$ 33 and a latent period of 40 $\pm$ 3 min for phage 2972. Larbi et al. (1990) observed similar latent periods (25 min) in two *S. thermophilus* phages, but their burst sizes of 56 and 88 particles per cell were considerably lower than those reported above. Recently, large burst sizes of 370 and 350 min, with latent periods between 30 and 40 min were reported by Guglielmotti et al. (2009b) for phages ALQ13.2 and ϕAbc2, respectively. All these phages are evidently able to multiply rapidly. Thus, the use of phage inhibitory media containing chelating agents (phosphates and citrates) might control phages that require Ca^{2+} to complete their lytic cycle (Suárez et al., 2002). However, some authors report that calcium ions are not absolutely required for *S. thermophilus* phage infection and that the completion of the lytic cycle is only partially retarded in absence of the cation (Binetti, 2001; Quiberoni, Guglielmotti, Binetti, & Reinheimer, 2004a).

These unique characteristics (heat resistance, rapid multiplication, and cation independence) restrict the development of strategies to reduce phage infections of *S. thermophilus* strains in industrial environments. Phages with these characteristics can

Table 1
Geographical origin and basic features of *Streptococcus thermophilus* bacteriophages.[a]

Country	Phages	Nature	Capsid (nm)	Tail length (nm)	Particulars	Sources
Argentina (110)	Ac1-4, BYM, FcSth3, O21-5, YAB, YEC; 53 others	Virulent	50–71	210–330 × 7–15		Suárez et al., 2002
	ϕ1 to ϕ11	Virulent	51–71	210–330 × 7–15		Quiberoni et al., 2003
	ϕ021-5, 10 other phages		40–70	175–300	Rosettes	Binetti et al., 2005
	ALQ1.3, 9 other phages	Virulent	61	298 × 8	Base plate	Quiberoni et al., 2006
	ϕLy1, 18 other phages	Virulent	- - -	- - -		Guglielmotti et al., 2009a
Canada (6)	DT1	Virulent	60	260 × 8		Tremblay & Moineau, 1999
	DT1, DT2, DT4	Virulent	60	260 × 8		Tremblay, 1999
	DT4, MD1, MD2, DT2, MD4	Virulent	- - -	- - -		Duplessis & Moineau, 2001
Denmark	1863	Virulent	- - -	- - -		Lévesque et al., 2005
Finland (10)	FKPStr10		50–56	217–239 × 4–8		Sarimo & Moksunen, 1978
	FA101, FK10, FK11, FK074, FKU101, FLO140/76, FLOThs, Fy101		50–56	217–239 × 4–8 or 215–260 × 5–6	1 Long fibre	Sarimo & Moksunen, 1978; Kivi et al., 1987
	FEThs		54–60	215–260 × 5–7	1 Long fibre	Kivi et al., 1987
France (65)	- -		60	240 × 8	1 Long fibre	Gélin, Wurch, & Linder, 1970
	Φ17	Virulent	42	215		Benbadis et al., 1990
	Φ57	Virulent	49	209	1 Long fibre	Benbadis et al., 1990
	ϕ80, 22 others	Virulent	55–58	221–275 × 8	1 Long fibre	Prévots, Relano, Mata, & Ritzenthaler, 1989
	ϕA1.1, ϕB1.2		50	246		Larbi et al., 1990
	0701, 10 other phages	Temperate	45–65	220–245	1 Long fibre	Fayard et al., 1993
	O1205 (synonym ϕO1205)	Temperate	69	260	Rosettes	Fayard et al., 1993; Le Marrec et al., 1997; Stanley et al., 1997
	2972	Virulent	55	260		Lévesque et al., 2005
	2139, 2331, 2217, 2756, 1433	Virulent	- - -			Lévesque et al., 2005
	858	Virulent	- - -	- - -		Barrangou et al., 2007
	SFi11, ST12	Virulent	65	230–260	1 Long fibre	Brüssow et al., 1994; Lucchini et al., 1998
	SFi21 (synonym ϕSFi21)	Temperate	65	230–260		Brüssow et al., 1994
	S3 (synonym ϕS3)	Virulent	65	230–260	Rosettes	Brüssow et al., 1994, 1998
	SFi2, SFi3J, SFi18, SFi19, ST44, ST44A	Virulent	65	230–260		Brüssow et al., 1994
	c20, st2, 447-B4, Φ1, BaS19, BaS265, Φ83, Φ117	Virulent	- - -	- - -		Le Marrec et al., 1997
Gabon	2768	Virulent	- - -	- - -		Lévesque et al., 2005
Germany (63)	P6, P55, PSt 124/44	Virulent	- - -	- - -	1 Long fibre	Neve et al., 1989
	P0, P8, P53, a10/J9, 50/34, 55/15, 71/45, 71/ST15	Virulent	54 × 61	224 × 11–12	1 Long fibre	Neve et al., 1989, Krusch et al., 1987
	P1, 50 other phages		54 × 61	224 × 11–12	1 Long fibre	Krusch et al., 1987
	TP-34	Temperate	- - -	240–260	1 Long fibre	Neve et al., 2003
Italy (57)	Φ 18	Temperate	57	234	Rosettes	Carminati & Giraffa, 1992
	Φ31, Φ33, Φ45, Φ47	Temperate	- - -	- - -		Le Marrec et al., 1997
	ϕSt21, 9 other phages	Virulent	- - -	- - -		Zago et al., 2003
	404 g, 7 other phages	Virulent	- - -	- - -		Rajagopal & Sandine, 1989
	S11, S17, S19, S69, S89, S96, M4-15, M4-31	Virulent	65	230–260		Brüssow et al., 1994
	S1, S4, S5, S6, S7, S8, S9, S12, S13, S15, S16, S18, S55, S66, S77, S88, S94, S95, S97, M1-5, M2-7, M2-8, M2-31, M2-49, M3-20, M4-29	Virulent	- - -	- - -		Brüssow et al., 1994
Portugal	1436	Virulent	- - -	- - -		Lévesque et al., 2005
Russia (2)	- -		40	220 × 8		Koroleva, Bannikova, Mytnik, & Bespalova, 1978
	- -		65	420 × 8		Koroleva, Bannikova, Mytnik, & Bespalova, 1978
Switzerland (11)	19S		60	255 × 12		Bauer, Dentan, & Sozzi, 1970
	24		55	255 × 12	1 Long fibre	Bauer et al., 1970
	s265		- - -	- - -		Sozzi & Maret, 1975
	——		- - -	- - -	1 Long fibre	Sozzi, 1977
	b6		49	209 × 9		Accolas & Spillmann, 1979
	c20-1		50	213 × 8		Accolas & Spillmann, 1979
	c20-2		50	130 × 8	Fibrous mass	Accolas & Spillmann, 1979
	sc		53	208 × 9	Rosettes	Accolas & Spillmann, 1979
	sch		50	200 × 9	Rosettes	Accolas & Spillmann, 1979
	sf		50	224 × 9		Accolas & Spillmann, 1979
	st2		52	200 × 9		Accolas & Spillmann, 1979

(*continued on next page*)

Table 1 (*continued*)

Country	Phages	Nature	Capsid (nm)	Tail length (nm)	Particulars	Sources
The Netherlands (10)	Φ 7201					Le Marrec et al., 1997
	Q7, Q10	Virulent	- - -	- - -		Le Marrec et al., 1997
	Φ4FN, Φ8FN, Φ7203, Φ7205, Φ7206, Φ7209, P4	Virulent	- - -	- - -		Le Marrec et al., 1997
Tunisia	2332	Virulent	- - -	- - -		Lévesque et al., 2005
U.S.A. (7)	ST2		60	236 × 10		Reddy, 1974; Reinbold et al., 1982
	ST4		65	290 × 10		Reddy, 1974; Reinbold et al., 1982
	STX		60	222 × 11		Reddy, 1974; Reinbold et al., 1982
	Q1, Q3, Q6	Virulent	- - -	- - -		Le Marrec et al., 1997
	Q5		- - -	- - -		Duplessis & Moineau, 2001

[a] Numbers in parentheses after a country indicate the number of phages studied; two dashes (- -) indicate no name assigned to a phage; three dashes (- - -) indicate no dimensions given for a phage.

cause the breakdown of starter cultures, even if they are present in low numbers at the beginning of the fermentation process. Furthermore, phages may remain in the air for long periods, meaning that aerosolization is one of the main dispersal mechanisms for phage particles (Mullan, 2003; Verreault, Moineau, & Duchaine, 2008). All of these factors contribute to the problem of phage spread within a dairy factory. They explain why cheese making is more sensitive to phage infection. Indeed, it involves the use of raw or pasteurized milk, open fermentation vats and repeated handling of products by equipments, utensils, and staff. In contrast, yogurt production is a process in which the fermented product has minimal exposure to the factory environment and milk undergoes a heat treatment at 90 °C, which kills most phages (Atamer et al., 2009; Capra, Binetti, Mercanti, Quiberoni, & Reinheimer, 2009; Quiberoni, Guglielmotti, & Reinheimer, 2004b).

3. Bacteriophage names, morphology, and host range

Bacteriophage naming is not regulated by any conventions, although an agreement is presently debated (Kropinski, Prangishvili, & Lavigne, 2009). *S. thermophilus* phages are not exempt from freelance naming. For example, the Greek letter phi (ϕ) is inconsistently added to some phage names, preceding the name of bacteriophages or not. In this way, one and the same phage may be designated as ϕO1205 or simply as O1205 (Stanley, Fitzgerald, Le Marrec, Fayard, & van Sinderen, 1997). Similarly, phages S3, Sfi11 and Sfi21 are also called ϕS3, ϕSfi11, and ϕSfi21 (Brüssow et al., 1994, 1998). The creation of needless synonyms is a source of confusion and properly unacceptable in virology.

All phages of *S. thermophilus* are members of the *Siphoviridae* family and the *Caudovirales* order, characterized by isometric capsids and long, non-contractile tails (Table 1, Fig. 1). To date, neither *Myoviridae* nor *Podoviridae* phages have been reported in *S. thermophilus*. In a general way, this applies to the vast majority *Streptococcus* spp. phages reported in the literature (Ackermann, 2007) and must have phylogenetic reasons. Long fibres, tiny base plates or a fibrous mass are occasionally observed at the tail ends in a few *S. thermophilus* phages (Accolas & Spillmann, 1979, Brüssow et al., 1994). In some, agglutination of the phage tails can result in the formation of large rosettes of phage particles (Accolas & Spillmann, 1979; Binetti, del Rio, Martin, & Alvarez, 2005; Brüssow et al., 1994; Quiberoni et al., 2006; Reinbold, Reddy, & Hammond, 1982; Stanley et al., 1997). In phage ST_2, Reinbold et al. (1982) observed polytails four times longer than the normal tail and suggested that their formation depended primarily on the length of incubation time.

Phage dimensions are often imprecise if not downright dubious due to the use of different electron microscopes and the apparent absence of magnification control in most publications (Table 1). Phage capsids are generally about 60 nm in diameter, but abnormally small head diameters of 40–42 nm have been reported (Benbadis et al., 1990; Binetti et al., 2005). Phages fall primarily into two groups with respect to tail length, short tails of approximately 220 nm and long tails of 330 nm. A few rare phages have very short or very long tails of approximately 130 and 420 nm in length, respectively. They may represent damaged or aberrant particles. Tail lengths are dangerously imprecise when they report length ranges within phage groups, but without data for individual phages. These average descriptions are of little value if electron microscopy-based phage descriptions are based on the examination of crude lysates or non-purified phages without adequate magnification calibration (Ackermann, 2007).

S. thermophilus phages are clearly host-specific (Deane et al., 1953; Kiuru & Tybeck, 1955; Kivi, Peltomäki, Luomala, & Sarimo, 1987). Sozzi and Maret (1975) tested 23 strains of *S. thermophilus* against a phage s265, of which only one was susceptible to infection. Another study showed that phages from different countries had distinct host ranges and were generally unable to replicate in *S. thermophilus* strains from other countries (Krusch et al., 1987). Similar results were reported by

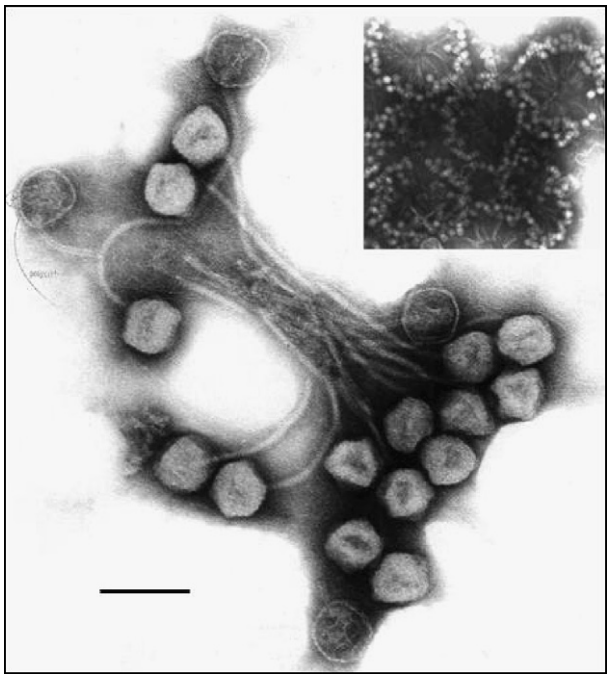

Fig. 1. *Streptococcus thermophilus* phage ALQ1.3 forming rosettes (insert). Final magnifications, 297,000× and 92,400× (insert); 2% phosphotungstate. The bar indicates 100 nm. Pictures were taken using a Philips EM 300 electron microscope operated at 60 kV (Département de Microbiologie, Infectiologie et Immunologie, Faculté de Médecine, Université Laval, Québec, Canada).

several authors (Benbadis et al., 1990; Zago, Carminati, & Giraffa, 2003), confirming the inadequacy of host range data for classification of *S. thermophilus* phages. Contrary to the observations of Brüssow et al. (1998), no correlation between DNA homology and host range was found in *S. thermophilus* phages (Benbadis et al., 1990; Le Marrec et al., 1997; Quiberoni et al., 2006).

Duplessis and Moineau (2001) characterized the antireceptor gene (*orf18*) of *S. thermophilus* phage DT1 and found that a variable region (VR2) within this gene is likely to be responsible for host specificity. Brüssow and Desiere (2001) suggested that *S. thermophilus* phages differing in host range have completely unrelated variable regions, whereas those with overlapping host ranges share highly related variable regions. Recent results (Binetti et al., 2005; Guglielmotti, Binetti, Reinheimer, & Quiberoni, 2009a) indicate that additional phage and host factors (such as resistance mechanisms) could be involved in the host specificity of these phages, as previously proposed by other authors (Duplessis & Moineau, 2001; Duplessis, Lévesque, & Moineau, 2006; Sturino & Klaenhammer, 2006). It is worth mentioning that no phage receptors at the host cell surface are known in *S. thermophilus*.

This host specificity is the backbone of the traditional starter culture rotation programs used in dairy plants to avoid an increase in phage concentration and likely subsequent fermentation failures. An efficient strain rotation program also requires a close monitoring of the phage diversity within a given dairy plant in order to control a newly emerged phage.

4. Bacteriophage genomics

S. thermophilus phages have double-stranded DNA genomes ranging in size from 29 to 43 kbp. Currently, 11 complete genomes of *S. thermophilus* phages, representing both *cos* and *pac*-types, are available in the public database of GenBank (NCBI). Genomic analysis has shown that all these phage genomes are similarly organized into distinct modular regions, the DNA replication and host lysis genes being the most conserved elements (Fig. 2). The difference between *cos* and *pac*-type phages (Fig. 2) is striking. The five *cos*-type phages for which the complete genome sequence is available, include the virulent phages DT1 (Tremblay & Moineau, 1999), 7201 (Le Marrec et al., 1997), Sfi19 (Lucchini, Desiere, & Brüssow, 1998), Abc2 (Guglielmotti et al., 2009b), and the temperate phage ϕSfi21 (Desiere, Lucchini, & Brüssow, 1998). The five *pac*-type *S. thermophilus* phages genome sequences are for the temperate O1205 (Stanley et al., 1997) and virulent phages Sfi11 (Lucchini et al., 1998), 2972 (Lévesque et al., 2005), 858 (Barrangou et al., 2007; Deveau et al., 2008), and ALQ13.2 (Guglielmotti et al., 2009b). The genome of *S. thermophilus* phage 5093 (Mills et al., 2009) was recently made available through GenBank. According to our bioinformatic analyses, this phage is different from the 10 others and may even represent a hybrid. Because the characterization of this phage has not been published yet, it will not be discussed in this review.

A comparison of the first 10 *S. thermophilus* phages shows that, like their hosts, they are rather homogeneous. It has been concluded that they belong to one polythetic species comprising both temperate and virulent phages (Brüssow & Desiere, 2001; Deveau et al., 2008). Thus, all *S. thermophilus* phages are related by DNA homology, albeit to different degrees (Brüssow et al., 1998). Comparative genome analyses led to not only the assessment of their relationships with other phages, but also point to mechanisms responsible for their diversity (Canchaya, Proux, Fournous, Bruttin, & Brüssow, 2003). Diversification is driven by point mutations, gene disruption, and recombination events (Deveau et al., 2008). In addition, transcriptomes are available for four phages, namely Sfi21 (Ventura, Bruttin, Canchaya, & Brüssow, 2002a; Ventura et al., 2002b), Sfi19 (Ventura & Brüssow, 2004), DT1, and 2972 (Duplessis, Russell, Romero, & Moineau, 2005).

For the purpose of this review, we have investigated the presence of a core genome in *S. thermophilus* phages. A core genome is defined as a set of genes invariably present and conserved in a group of isolates (Tettelin et al., 2005). A gene is considered conserved when two proteins can be aligned with a minimum of 50% amino acid sequence conservation over 50% of the protein length (Muzzi, Masignani, & Rappuoli, 2007; Rousseau & Moineau, 2009). Using these definitions, a core genome was individualized within 10 phage genomes (Fig. 2). This core genome is small and comprises either four ORFs (phages O1205, Sfi11, Sfi19, Sfi21, 7201, Abc2) or five ORFs (phages 858, 2972, ALQ13.2, DT1). The fifth ORF in the last four phages is a duplicate endolysin gene. Using phage DT1 as a model, these conserved ORFs include ORF21 (unknown function), ORF25 (endolysin), ORF27 (endolysin), ORF42 (a putative DNA-binding protein), and ORF44 (unknown function).

In addition, we established the core genomes for *cos*-type and *pac*-type phages. In the five *cos*-type viruses, 26 ORFs (phages 7201, Sfi19, Sfi21) or 27 ORFs (phages DT1 and Abc2) were conserved and considered as core genomes. The additional ORF in phages DT1 and Abc2 was again the endolysin. Using phage DT1 as a model for gene designation, this core includes ORF2, ORF4 to ORF6, ORF8 to ORF15, ORF17 to ORF19, ORF21, ORF22, ORF24, ORF25/ORF27 (endolysin), ORF39, ORF40, ORF42, ORF43 to ORF46 (Fig. 2).

Within the five *pac*-type genomes, a total of 24 ORFs (phages O1205 and Sfi11), 25 ORFs (phage ALQ13.2) or 26 ORFs (phages 2972 and 858) form another core genome. The differences in the number of ORFs are due to presence or absence of the endolysin and the large terminase subunit. Using phage 2972 as a model, this core includes ORF3/ORF4 (terminase), ORF5 to ORF19 (morphogenesis module), ORF21, ORF22, ORF26/ORF29 (endolysin), ORF35, ORF37, ORF38, ORF42, and ORF44 (Fig. 2).

In conclusion, only a few ORFs are conserved within all *S. thermophilus* phages, while both *cos*-type and *pac*-type phages,

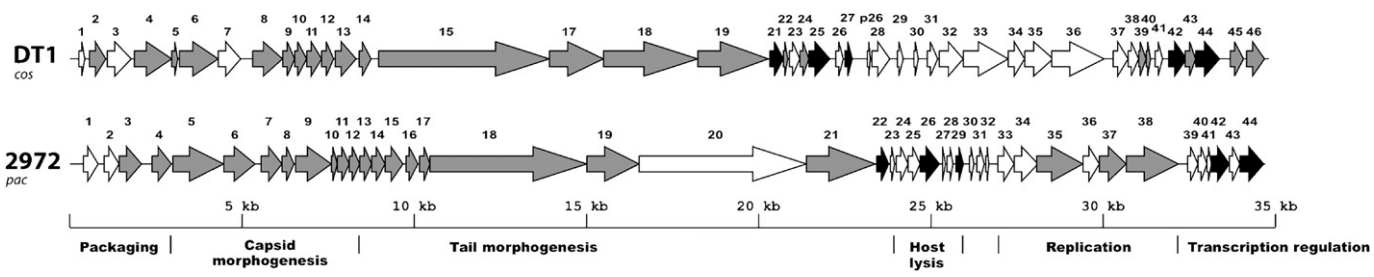

Fig. 2. Schematic representation of *S. thermophilus* bacteriophage core genomes. Each line represents a different phage genome and each arrow represents a putative protein. DT1 is used here as the model phage for the *cos*-type while phage 2972 is used for the *pac* type. ORFs in black illustrate the core genome of all *S. thermophilus* phages for which the complete genome is available (O1205, Sfi11, 858, 2972, ALQ13.2, DT1, Sfi19, Sfi21, 7201, and Abc2). ORFs in grey represent the core genome of the *cos*-type (DT1, Sfi19, Sfi21, 7201 and Abc2) and the *pac*-type (O1205, Sfi11, 858, 2972 and ALQ13.2), respectively.

respectively, share numerous genes. They are considered as two subspecies, each being neatly characterized by a set of specific genes. The core genomes include genes responsible for key phage functions. It remains to be seen if these cores will be upheld as more *S. thermophilus* phage genomes become available (Rousseau & Moineau, 2009).

5. Detection of *S. thermophilus* phages

Control of starter activity and plaque assays are routinely used to detect, count, and purify phages because they provide information not only on the sensitivity of a particular *S. thermophilus* strain, but also on the presence of ongoing virulent phage multiplication. In particular, the double-layer method (Adams, 1959) lends itself to the detection of individual phage plaques on a semi-solid agar layer incorporating a host strain, but its usefulness for detection of *S. thermophilus* phages has been reported to be variable and uncertain. Interactions between phage and host cells, growth medium, physical and chemical conditions of the double-agar plates are among the main factors affecting plaque formation and consequently, host range analyses (Mullan, 1979). Despite several attempts to optimize this method, the efficiency of phage detection ultimately depends on each phage–host system (Lillehaug, 1997). Even if the double-layer method is theoretically simple and easily applicable in any quality control laboratory, it is time-consuming and requires long incubation times, and its results may ultimately arrive too late to save whole vats of inoculated milk. This last disadvantage, coupled with the ever-increasing availability of phage genomic sequences, has prompted the development of several PCR-based techniques in order to overcome phage detection problems. Some of them were adapted to overcome the notorious difficulties in detecting microorganisms in milk samples by PCR (Binetti et al., 2005) or in milk-derived products such as whey (Brüssow et al., 1994).

The first PCR assay reported for *S. thermophilus* phage detection in dairy samples was based on the amplification of conserved DNA fragment in many, but not all, *S. thermophilus* phages of the Nestlé collection. This fragment covered part of *orf271* and *orf504* of phage Sfi11. (Brüssow et al., 1994). This target did not allow for phage identification within the two known groups. Binetti et al. (2005) developed a method to amplify VR2, the variable region of the antireceptor gene (Duplessis & Moineau, 2001), allowing both rapid detection and classification of *S. thermophilus* phages. A significant advantage of this method is the correlation with host ranges, thereby allowing a rational modification of the starter rotation system. The same authors then described a simple and rapid multiplex PCR method that detected, in a single reaction, the presence of phages infecting *S. thermophilus, Lactobacillus delbrueckii* and the three distinct groups of *L. lactis* phages commonly found in dairy plants (P335, 936 and c2) (del Río et al., 2007).

Another multiplex PCR-based method was developed to classify *S. thermophilus* phages into *cos*- or *pac*-types (Quiberoni et al., 2006), although it was not adapted to detect phages in dairy samples. The first *S. thermophilus* phage detection method based on fast real-time PCR technology was recently reported by del Río, Martín, Martínez, Magadán, and Álvarez (2008) to quantitatively detect and identify *cos*- and *pac*-type *S. thermophilus* phages in milk samples. The proposed qPCR procedure was reported to be sensitive and specific as well as allowing quantification and identification of the type of *S. thermophilus* phage in a short period of time. A limitation, however, is that these PCR-based methods need special equipment, stringent laboratory precautions, and expert personnel. Therefore, although many molecular methods for phage detection continue to emerge from research laboratories, it is still unclear whether these methods will find their way into routine use in the dairy industry (Brüssow et al., 1994).

6. Phage-resistant *S. thermophilus* strains

When a new virulent phage emerges, an industrial *S. thermophilus* strain may need to be replaced by either a new strain or by a phage-resistant mutant. The latter can be obtained by at least three means. The first method is the development of bacteriophage-insensitive mutants (BIMs) (Binetti, 2001; Mills et al., 2007; Viscardi et al., 2003a; Viscardi, Capparelli, & Iannelli, 2003b). They are spontaneous phage-resistant derivatives that survive long exposure to virulent phages, probably because of a modification in the (still unknown) receptor of the wild-type strain. This technique is strongly strain-dependent.

The second method is based on the improvement of the natural antiviral systems of the bacterial cell (Sturino & Klaenhammer, 2006). However, until recently, few phage exclusion systems had been reported in *S. thermophilus* (Moineau, 1999). For example, 11 restriction/modification systems were identified in various *S. thermophilus* strains, but proved to be relatively weak (for a review see Sturino & Klaenhammer, 2004b). A prophage gene coding for a surface lipoprotein was also found to confer superinfection exclusion by inhibiting phage DNA release into the cell (Sun, Göhler, Heller, & Neve, 2006). A powerful chromosomally-encoded antiphage system was recently found in most if not all *S. thermophilus* strains (Barrangou et al., 2007; Deveau et al., 2008; Horvath et al., 2008). Indeed, clustered regularly interspaced short palindromic repeats (CRISPR) and their associated *cas* genes are linked to a mechanism of acquired phage resistance. In *S. thermophilus*, CRISPR1 is formed of tandem arrays composed of 36-bp direct repeats separated by 30-bp non-repetitive DNA (spacers) (Barrangou et al., 2007). As indicated above, when a phage-sensitive strain of *S. thermophilus* is infected with a virulent phage, BIMs will emerge. Many of these BIMs have acquired at least one new repeat-spacer unit at the 5′ end of a CRISPR locus (Labrie, Samson, & Moineau, 2010). The newly added spacer is 100% identical to a sequence (named proto-spacer) found in the genome of the infecting phage. Interestingly, different BIMs can acquire distinct spacers from the same phage genome. Their only common feature is the presence of a short conserved nucleotide motif (e.g., NNAGAAW) flanking the proto-spacer in the phage genome, which is important for phage resistance (Deveau et al., 2008; Labrie et al., 2010). The readers interested in this system are directed to the following recent review (van der Oost, Jore, Westra, Lundgren, & Brouns, 2009).

The third way is genetic engineering (Sturino & Klaenhammer, 2006). Known antiphage systems from another bacterium (*Lactococcus*) can be introduced into *S. thermophilus* (Moineau, Walker, Holler, Vedamuthu, & Vandenbergh, 1995; Tangney & Fitzgerald, 2002). Antiphage mechanisms have also been engineered using *S. thermophilus* phage genetic elements such as a phage origin of replication (*ori*) located on a plasmid, which attracts phage replication factors (Foley, Lucchini, Zwahlen, & Brüssow, 1998; Lamothe et al., 2005; Stanley, Walsh, van der Zwet, Fitzgerald, & van Sinderen, 2000). Antisense RNA targeting a conserved phage gene was also used to confer phage resistance to *S. thermophilus* (Sturino & Klaenhammer, 2002; Sturino & Klaenhammer, 2004a). Subunit poisoning was cleverly designed to express *in trans* phage mutant proteins that suppressed the function of a native *S. thermophilus* phage primase (Sturino & Klaenhammer, 2007). To our knowledge, genetic engineering is not yet industrially used to induce phage resistance, but is exploited to gain insight into phage biology and phage–host interactions.

7. Concluding remarks

Table 1 suggests that research on *S. thermophilus* phages is highly biased towards the specific ecological niche of milk. Most, if not all, *S. thermophilus* phages have been isolated from this environment, which is also where its hosts are generally found. Nonetheless, *S. thermophilus* phages are clearly divided into two homogenous groups that are equally adapted to rapid multiplication during the milk fermentation process. This is likely due to the fact that only a limited number of performing *S. thermophilus* strains are used in industrial starter cultures around the world, leading to the emergence of closely related virulent phages. Although this is currently unknown, these strains may carry related phage receptors. These *S. thermophilus* hosts are also relatively homogeneous with a low level of polymorphism (Bolotin et al., 2004; Hols et al., 2005), suggesting a recent emergence of this bacterial species and a constant evolution towards a specialized bacterium dedicated to growth in milk. All available data support the current classification of *S. thermophilus* phages, regardless the type of dairy samples or their geographical origin, on the basis of their DNA packaging mechanism (*cos* and *pac*) and structural protein composition.

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