International Journal of Food Microbiology 177 (2014) 89–97

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Response of *S. thermophilus* LMD-9 to bacitracin: Involvement of a BceRS/AB-like module and of the rhamnose–glucose polysaccharide synthesis pathway

B. Thevenard [a,b,1], C. Besset [a,b], S. Choinard [a,b,2], P. Fourcassié [c], P. Boyaval [c], V. Monnet [a,b], F. Rul [a,b,*]

[a] INRA, UMR1319 Micalis, F-78350 Jouy-en-Josas, France
[b] AgroParisTech, UMR1319 Micalis, F-78350 Jouy-en-Josas, France
[c] DuPont Nutrition and Health, Danisco France, BP10, 386220 Dangé-Saint-Romain, France

ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form 3 February 2014
Accepted 15 February 2014
Available online 22 February 2014

Keywords:
Antibiotic
Detoxification modules
Rhamnose–glucose polysaccharide
Streptococcus thermophilus
Two-component systems
Yogurt

ABSTRACT

Streptococcus thermophilus is a lactic acid bacterium of major importance to the dairy industry as it is found in numerous cheeses and is one of the two bacterial species involved in the fermentation of yogurt. Bacterial two-component signal transduction systems (TCSs) play important roles in the process of bacterial environmental adaptation. *S. thermophilus* LMD-9 possesses eight such TCS systems; however, their functions have thus far been only poorly investigated. Here, we focused on two of the TCSs in LMD-9, TCS06 and TCS07, whose encoding genes are located close to each other on the chromosome, and are associated with those of ABC transporters. TCS06 homologs are frequently found in Lactobacillales, but their function has not yet been determined, while TCS07 and its upstream potential ABC transporter are homologous to the BceRS/AB system, which is involved in bacitracin resistance in *Bacillus* and *Streptococcus* species. To investigate the function(s) of TCS06 and TCS07, we constructed and characterized deletion mutants and performed transcriptional analysis in the presence and absence of bacitracin. We show here that both TCS06 and TCS07 regulate the genes in their close vicinity, in particular those encoding ABC transporters. We propose that the response of *S. thermophilus* to bacitracin includes i) a bacitracin export system, regulated by TCS07 and constituting a BceRS/AB-like detoxification module, and ii) the modification of cell-envelope properties via modulation of rhamnose–glucose polysaccharide synthesis, at least partially regulated by TCS06.

1. Introduction

Streptococcus thermophilus is a lactic acid bacterium of major importance in the dairy industry as it is found in numerous cheese microbial ecosystems and, with its partner *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*), represents the species responsible for the fermentation of yogurt. *S. thermophilus* has a long, documented history of safe use in foods, is a "generally recognized as safe" (GRAS) organism, and is on the European Qualified Presumption of Safety (QPS) list of food bacteria (EFSA, 2011). Over the course of its adaptation to milk, *S. thermophilus* has lost many genes linked to virulence and sugar utilization, but it has kept the essential gene sets involved in functions necessary for growth in milk, such as nitrogen metabolism (Bolotin et al., 2004) and utilization of lactose, the main sugar in milk.

In the food ecosystems in which *S. thermophilus* is used, it has to compete with other microorganisms for nutrients and space in order to reach the final population size that is required to make yogurt (i.e. 10^7 CFU/g). In this context, the ability to produce bacteriocins or to resist the bacteriocins produced by others confers a competitive advantage. Towards this end, *S. thermophilus* LMD-9 possesses a bacteriocin, thermophilin 9, that inhibits the growth of most species closely related to *S. thermophilus*, such as *Streptococcus salivarius*, *Enterococcus faecalis*, *Lactococcus lactis*, some *Listeria* (Fontaine et al., 2007; Fontaine and Hols, 2008), and *Pediococcus acidilactici* (Renye and Somkuti, 2013).

Bacterial two-component signal transduction systems (TCSs) play important roles in bacterial environmental adaptation, the production of virulence factors, self-defense, and biofilm formation (Stock et al., 2000). A typical TCS consists of a sensor histidine kinase (HK) – most often membrane bound – and a cytoplasmic response regulator (RR). Upon the reception of a signal, the sensor kinase undergoes autophosphorylation (at a conserved His residue). The phosphate group is then transferred to the cognate response regulator (at a conserved Asp residue), which activates or represses its target genes by binding to their promoter regions (Laub and Goulian, 2007). *S. thermophilus* LMD-9 possesses eight such TCSs (Hols et al., 2005), six complete with both HK and RR (TCS02, 04, 05, 06, 07, 09) and two orphan response regulators (RR01 and RR08). We have previously shown, using a

* Corresponding author at: INRA, UMR1319 Micalis, F-78350 Jouy-en-Josas, France.
E-mail address: francoise.rul@jouy.inra.fr (F. Rul).
[1] Present address: Yoplait Center for Development, 38205 Vienne Cedex, France.
[2] Present address: UMR1318 INRA-AgroParisTech, 78026 Versailles Cedex, France.

http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.011
0168-1605/

proteomic approach, that the proteins RR01 and RR05 are present after the growth of *S. thermophilus* LMG18311 in milk in the presence and absence of *L. bulgaricus* (Herve-Jimenez et al., 2008, 2009), as well as in bacteria recovered from the feces of *S. thermophilus* LMD-9 mono-associated rats (Rul et al., 2011). We have also observed that the eight LMD-9 response regulator (*rr*) genes are expressed at different levels throughout growth in milk (Thevenard et al., 2011). Additionally, when *S. thermophilus* occurs in a mixed culture with *L. bulgaricus*, expression of *rr01*, *rr02*, *rr05*, and *rr09* increases compared to mono-culture, suggesting that *S. thermophilus* detects the other bacterium's presence and initiates regulatory responses. These data suggest that TCSs could be involved in the adaptation of *S. thermophilus* to the diverse environments in which it is found along the food chain (e.g., dairy products or the digestive tract after product ingestion), each of which with its own various physiochemical and biotic constraints.

Bacterial signal transduction systems and the neighboring ABC transporters that they regulate often contribute jointly to adaptive responses to environmental changes. In some species, they have co-evolved to form specific detoxification modules. One such system is BceRS/AB, which is dedicated to the detection of and response to the antibiotic bacitracin in *Bacillus subtilis* (Ohki et al., 2003) and *Streptococcus mutans* (Ouyang et al., 2010). Bacitracin is a narrow-spectrum, non-ribosomal cyclic polypeptide antibiotic produced by *Bacillus* species (Johnson et al., 1945; Azevedo et al., 1993) that is directed primarily against gram-positive cocci and bacilli, including *Staphylococcus*, *Streptococcus*, and *Clostridium difficile*, as well as some Archaea (Ming and Epperson, 2002). Its primary mechanism of action is the inhibition of peptidoglycan synthesis: it blocks cell wall biosynthesis by binding to the membrane acceptor undecaprenyl pyrophosphate (UPP), inhibiting its dephosphorylation and thus disrupting the regeneration of undecaprenyl phosphate (UP) (Stone and Strominger, 1971).

Three out of the eight TCSs of *S. thermophilus* LMD-9 present complete ABC transporters (ATPase and permease present) in their close vicinity (TCS06, TCS07, and TCS09; Thevenard et al., 2011). Among these three TCSs, only the relationship between TCS09 and its ABC transporter has been previously characterized (Fontaine et al., 2007). TCS06 and TCS07 are physically close to each other (10 kb between the two loci) and are close neighbors of potential ABC transporters that are oriented in the same direction. TCS06 homologs are frequently found in Lactobacillales (Thevenard et al., 2011), but their function has not yet been determined, while TCS07 and its upstream potential ABC transporter are homologous to the BceRS/AB system of *B. subtilis*. Here, we show that TCS06 and TCS07 regulate the genes in their close vicinity, in particular those encoding ABC transporters, and that expression of both TCSs is modulated by bacitracin, which was used here as a model for antibiotic peptide stress. Our data demonstrate that the response of *S. thermophilus* LMD-9 to bacitracin involves TCS07 and its adjacent ABC transporter, probably constituting a four-component detoxification module similar to BceRS/AB. The rhamnose–glucose polysaccharide (RGP) synthesis pathway is also involved in this antibiotic stress response, possibly in part through direct or indirect regulation via TCS06. Several pathways and regulators thus participate in the response of *S. thermophilus* to bacitracin, involving the export system and probably cell envelope modifications.

2. Material and methods

2.1. Bacterial strains, plasmids, and media

The bacterial strains used are listed in Table 1. *E. coli* TG1 RepA+ was grown in LB medium, with shaking, at 37 °C. *S. thermophilus* was grown in 1% (wt/vol) lactose M17 (M17Lac) broth (DIFCO) at 42 °C. When required, erythromycin (250 μg/mL for *E. coli*; 2.5 μg/mL for *S. thermophilus*) or kanamycin (30 μg/mL for *E. coli*; 1 mg/mL for *S. thermophilus*) was added to the medium. Stock cultures of *S. thermophilus* strains grown in chemically defined medium (CDM; Letort and Juillard, 2001) were prepared from 8-h cultures kept at 42 °C that were inoculated with one isolated M17Lac-agar clone.

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Relevant properties	References
Bacterial strains		
S. thermophilus		
LMD-9	Wild-type	Makarova et al. (2006)
TIL1189	LMD-9 Δ*rr06*	Thevenard et al. (2011)
TIL1190	LMD-9 Δ*rr07*	Thevenard et al. (2011)
TIL1422	LMD-9Δ*rmlC*, Kana^R	This study
TIL1361	LMD-9 Δ*STER_0656*, Ery^R	This study
TIL1446	LMD-9 Δ*STER_1307* Δ*STER_1308*, Kana^R	This study

2.1.1. Effect of bacitracin on S. thermophilus survival

Wild-type and mutant strains were grown in CDM in the presence (0.1, 0.2, or 0.4 μg/mL) or absence of bacitracin. We measured $OD_{600\ nm}$ after 24 h of growth and calculated the ratio of OD when bacitracin was present /OD when bacitracin was present for the three bacitracin concentrations tested.

2.2. Construction of mutants ΔrmlC, ΔSTER_0656 (rmlC-like), and ΔSTER_1308ΔSTER_1307

Negative mutants were constructed by gene interruption with kanamycin or erythromycin resistance cassettes using three fragments PCR that were the Kana or Ery cassette itself as well as an upstream and a downstream PCR fragment (Table 2). The 3′ end of the upstream-generated fragments contained a sequence complementary to the 5′ end of the Kana or Ery cassettes whereas the 5′ end of the downstream-generated fragments contained sequences complementary to the 3′ end of the cassettes. The joining of the three fragments (upstream, cassette, and downstream) for each mutant was performed via PCR using primers UP1223F/DN1223R, UP656F/DN656R, UP1293F/DN1292R, and UP1308F/DN1307R under the following cycling conditions: Taq polymerase (Taq Phusion) activation at 98 °C for 3 min, followed by 35 cycles of 30 s at 97 °C, 30 s at 55 °C, and 4 min at 72 °C. After purification with a QIAquick PCR purification kit, 500 ng of each of the resulting fragments was used to transform LMD-9 natural competent cells as described by Ibrahim et al. (2007). Transformants were selected on M17Lac plates using kanamycin (1 mg/mL) or erythromycin (2.5 μg/mL) and were then checked by PCR using oligonucleotides AphA3-2/DN1223R, UP656F/Erm1, UP1293F/Erm1, and AphA3-2/DN1307R. Finally, sequencing of the flanking regions was performed to ensure that no unwanted mutations had been introduced.

2.3. Scanning electron-microscopy (SEM) and transmission electron-microscopy (TEM) analyses

All microscopy analyses were performed on the MIMA2 imaging platform (INRA, http://www6.jouy.inra.fr/mima2/).

For SEM observations, one 40-μL-drop of fresh CDM liquid culture (collected at log phase; $OD_{600\ nm} = 0.5$) was deposited onto a sterile aluminum coupon (10-mm diameter, sterilized just before use by sonication in ethanol and UV exposure). Each coupon was placed into one well of a 24-well polystyrene plate. Sedimentation lasted 3 h. Samples were fixed via careful immersion in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 and room temperature (RT); they remained immersed for 4 h. They were then washed three