



# YMC-2011, a Temperate Phage of *Streptococcus salivarius* 57.I

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**ABSTRACT** *Streptococcus salivarius* is an abundant isolate of the oral cavity. The genome of *S. salivarius* 57.I consists of a 2-Mb chromosome and a 40,758-bp circular molecule, designated YMC-2011. Annotation of YMC-2011 revealed 55 open reading frames, most of them associated with phage production, although plaque formation is not observed in *S. salivarius* 57.I after lytic induction using mitomycin C. Results from Southern hybridization and quantitative real-time PCR confirmed that YMC-2011 exists extrachromosomally, with an estimated copy number of 3 to 4. Phage particles were isolated from the supernatant of mitomycin C-treated *S. salivarius* 57.I cultures, and transmission electron microscopic examination indicated that YMC-2011 belongs to the *Siphoviridae* family. Phylogenetic analysis suggests that phage YMC-2011 and the *cos*-type phages of *Streptococcus thermophilus* originated from a common ancestor. An extended  $-10$  element ( $p_L$ ) and a  $\sigma^{70}$ -like promoter ( $p_R$ ) were mapped 5' to *Ssal*\_phage00013 (encoding a CI-like repressor) and *Ssal*\_phage00014 (encoding a hypothetical protein), respectively, using 5' rapid amplification of cDNA ends, indicating that YMC-2011 transcribes at least two mRNAs in opposite orientations. Studies using promoter-chloramphenicol acetyltransferase reporter gene fusions revealed that  $p_R$ , but not  $p_L$ , was sensitive to mitomycin C induction, suggesting that the switch from lysogenic growth to lytic growth was controlled mainly by the activity of these two promoters. In conclusion, a lysogenic state is maintained in *S. salivarius* 57.I, presumably by the repression of genes encoding proteins for lytic growth.

**IMPORTANCE** The movement of mobile genetic elements such as bacteriophages and the establishment of lysogens may have profound effects on the balance of microbial ecology where lysogenic bacteria reside. The discovery of phage YMC-2011 from *Streptococcus salivarius* 57.I suggests that YMC-2011 and *Streptococcus thermophilus*-infecting phages share an ancestor. Although *S. salivarius* and *S. thermophilus* are close phylogenetically, *S. salivarius* is a natural inhabitant of the human mouth, whereas *S. thermophilus* is commonly found in the mammary mucosa of bovine species. Thus, the identification of YMC-2011 suggests that horizontal gene transfer via phage infection could take place between species from different ecological niches.

**KEYWORDS** *Streptococcus salivarius*, temperate phage, lysogeny, plasmid, *Siphoviridae* family

Phages, prokaryotic viruses, are the most abundant biological entities on the planet (1). Although phages have been isolated from the oral cavity (2, 3), relatively little is known about the phages of oral streptococci, compared to those of other lactic acid bacteria, particularly *Lactococcus lactis* and *Streptococcus thermophilus*. To date, the better characterized phages of oral streptococci are the virulent phage M102AD of *Streptococcus mutans* (4) and the temperate phage SM1 of *Streptococcus mitis* (5). A *Streptococcus salivarius*-infecting phage of the family *Cystoviridae* was described re-

Received 21 November 2016 Accepted 4 January 2017

Accepted manuscript posted online 6 January 2017

**Citation** Chou W-C, Huang S-C, Chiu C-H, Chen Y-YM. 2017. YMC-2011, a temperate phage of *Streptococcus salivarius* 57.I. *Appl Environ Microbiol* 83:e03186-16. <https://doi.org/10.1128/AEM.03186-16>.

**Editor** M. Julia Pettinari, University of Buenos Aires

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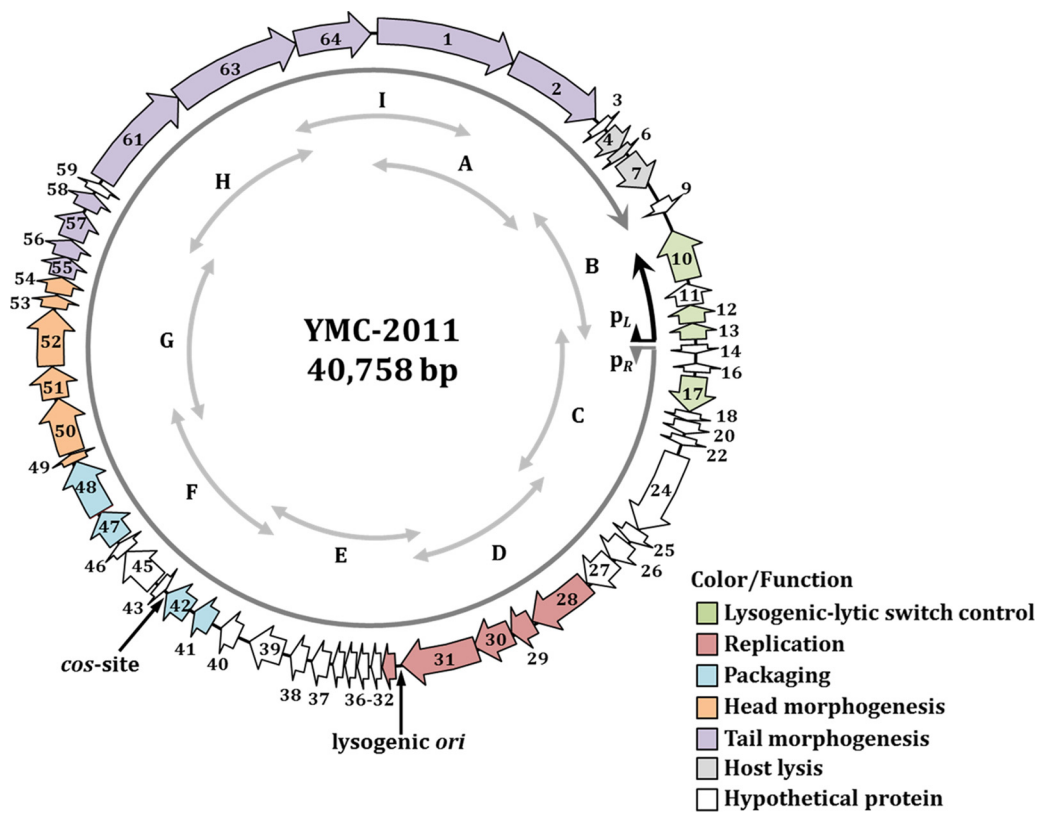
cently (6), but that phage has not been characterized. Although prophages have been found in the genomes of *S. salivarius* strains JF, NCTC8618, and JIM8777 (7), phage production has not been reported. In contrast, more than 300 virulent and temperate phages have been observed in *S. thermophilus* (8), a close relative of *S. salivarius*. Analyses indicate that all *S. thermophilus*-infecting phages are from a common ancestor, with a hexagonal capsid and a long noncontractile tail (9), belonging to the *Siphoviridae* family (10). *S. thermophilus*-infecting phages have been commonly classified as *cos*-type or *pac*-type phages based on their DNA-packaging machinery and structural proteins (11). For instance, the virulent phages DT1 (12), 7201 (11), Sfi19 (13), and Abc2 (14), as well as the temperate phage Sfi21 (15), are *cos*-type phages, whereas the temperate phages O1205 (11) and TP-J34/TP-778L (16) and the virulent phages Sfi11 (17), 2972 (18), 858 (19), and ALQ13.2 (14) are *pac*-type phages. Additional groups, including phage 5093 (20) and the newly described 987 phage group (21), are classified independently, as both possess mosaic genomes, presumably resulting from a relatively recent horizontal gene transfer and/or recombination event. Similarly, two DNA replication modules, represented by phages Sfi21 and 7201, have been observed with *S. thermophilus*-infecting phages; however, most such phages possess the conserved Sfi21-like replication module (22).

Temperate phages usually exist in a latent form but can become lytic. In the lysogenic cycle, the phage genome is either integrated into the host chromosome or exists as a low-copy-number plasmid. For example, P1 of *Enterobacteriaceae* (23) and pZL12 of *Streptomyces* spp. (24) exist as closed circular plasmids in the cognate host. During lysogenic growth, the bacterial host grows normally, and no phage particle is produced. When the survival of the lysogen is threatened by harmful growth conditions, the prophage is released from the chromosome and exists as a plasmid, and DNA replication begins (25). As seen for phage  $\lambda$  and many other temperate phages, the genetic switch from lysogenic growth to lytic growth is generally controlled by the availability of two phage-encoded transcription factors, CI and Cro. Genes encoding these two proteins are arranged in opposite orientations, and the presence of the transcription factor inhibits transcription from the opposite promoter by binding to specific operators, thereby establishing lysogenic growth. When the SOS response is activated in a  $\lambda$  lysogen, the extensive DNA damage activates RecA, which acts as a corepressor to stimulate specific cleavage of CI, leading to lytic growth (26). Upon entering lytic growth, the phage genome replicates autonomously, head and tail proteins are synthesized, the phage genome is packaged, and the phage particles are released when the host cells lyse.

Advances in genomic analysis have allowed for the identification of previously unknown elements. The complete genome sequence of *S. salivarius* strain 57.I reveals a chromosome and a 40,758-bp plasmid, designated YMC-2011 (27). Most of the open reading frames (ORFs) annotated from YMC-2011 share significant homology with ORFs related to phage production, suggesting that strain 57.I is lysogenically infected with a temperate phage. In this study, we found that phage particles could be isolated from mitomycin C-treated *S. salivarius* 57.I cultures, although plaque formation was not detected.

## RESULTS

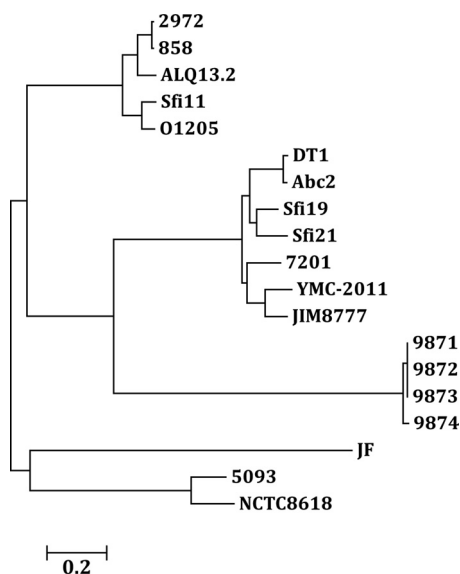
**Sequence analysis of YMC-2011.** Analysis of the YMC-2011 sequence (GenBank accession number [CP002889.1](https://www.ncbi.nlm.nih.gov/nuccore/CP002889.1)) (27) revealed that YMC-2011 has a GC content of 41% and encodes a total of 55 ORFs (Fig. 1), the basic characteristics of which are summarized in Table S1 in the supplemental material. Of particular note, the original designations Ssal\_phage00005, Ssal\_phage00008, Ssal\_phage00015, Ssal\_phage00019, Ssal\_phage00021, Ssal\_phage00023, Ssal\_phage00044, Ssal\_phage00060, and Ssal\_phage00062 are not identified as ORFs in the database. Twenty-four of the ORFs encode hypothetical proteins, and 10 of them are without homology in the nonredundant GenBank database. The remaining ORFs share significant levels of homology, at the deduced amino acid level, with ORFs found in *S. thermophilus* phages, including



**FIG 1** Schematic representation of the organization of YMC-2011. The relative location and orientation of each *Ssal*\_phage ORF are shown. ORFs that participate in the same pathway for phage production are in the same color. ORFs in white are of unknown function. The locations of the putative *ori* and *cos* site are indicated by arrows. The relative orientation and location of the two transcripts generated from  $p_L$  and  $p_R$  are indicated. The locations of the amplicons (A to I) presented in Fig. 3B are indicated by double-headed arrows.

those encoding structural proteins, transcriptional regulators governing the lysogenic-lytic switch, and enzymes for integration, DNA replication, packaging, and host cell lysis, indicating that YMC-2011 likely produces phage particles.

Two potential *ori* regions, with AT contents of >70%, were identified using Ori-Finder. The first region (nucleotides 9928 to 10078), located between *Ssal*\_phage00013 (encoding a *Ci*-like protein) and *Ssal*\_phage00014 (encoding a hypothetical protein), has an AT content of 73% and contains 3 putative DnaA boxes (with no more than 2 mismatches with respect to the consensus sequence 5'-TTWTSCAMA). The second region (nucleotides 19816 to 20057), located between *Ssal*\_phage00031 (encoding a primase) and *Ssal*\_phage00032 (encoding a phage protein), has an AT content of 71% and contains 2 putative DnaA boxes. Most of the characterized *ori* sequences in *S. thermophilus* phages are located in noncoding regions close to genes encoding proteins involved in DNA replication (18), suggesting that the region between *Ssal*\_phage00031 and *Ssal*\_phage00032 (region 2) contains the putative *ori* of YMC-2011. This hypothesis is supported by the sequence similarity between this region and the putative *ori* sequences of *S. thermophilus* phages belonging to the Sfi21 replication module. Specifically, significant sequence homology was found between region 2 and the proposed *ori* sequences of phages ALQ13.2 (14), O1205 (28), DT1 (12), and 2972 (18) (Fig. S1A). Furthermore, *ori* sequences are known to contain repeat regions (29) and, indeed, two sets of direct repeats (5'-GTTACCKT and 5'-TAAATAAA) and one imperfect inverted repeat (5'-TTATTTATATATT-N<sub>5</sub>-AATAAATAAATAA) were found in region 2 (Fig. S1B). A putative *cos* site, 5'-CCGCCACAAGGTG, was also identified 30 bases 3' to the TTG of *Ssal*\_phage00042 (encoding a terminase small subunit), based on comparisons with other known *cos* sequences. This *cos* site is highly homologous to the *cos* sites of phages 7201, Abc2, DT1, Sfi19, and Sfi21 (Fig. S2).

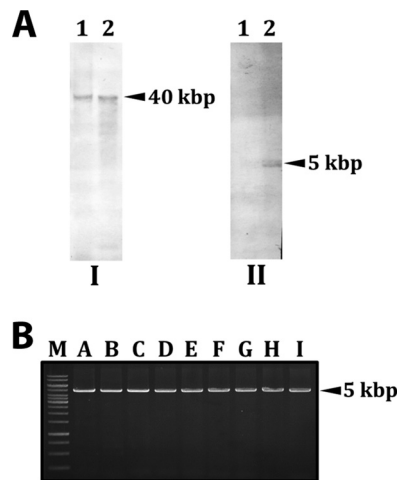


**FIG 2** Phylogenetic tree of YMC-2011 and other streptococcal phages. The whole-genome sequences of phages 2972, 858, ALQ13.2, Sfi11, O1205, DT1, Abc2, Sfi19, Sfi21, 7201, 9871, 9872, 9873, 9874, and 5093 of *S. thermophilus*, the prophages extracted from the genomes of *S. salivarius* strains JIM8777, JF, and NCTC8618, and phage YMC-2011 of *S. salivarius* 57.I were used to construct the tree. The tree was built using MEGA 6.0.

Based on the results of the homology analysis (Table S1), 15 *S. thermophilus*-infecting phages, 3 putative *S. salivarius* prophages, and phage YMC-2011 were selected for phylogenetic analysis (Fig. 2). As expected, the *cos*-type phages DT1, Abc2, Sfi19, Sfi21, and 7201 are in one cluster, and the *pac*-type phages 2972, 858, ALQ13.2, Sfi11, and O1205 are in a separate cluster. The newly identified 987 group (phages 9871, 9872, 9873, and 9874) is in a separate cluster. The prophages of *S. salivarius* (strains JIM8777, JF, and NCTC8618) and phage YMC-2011 are relatively distant from each other, with the exception of the prophage of strain JIM8777 and phage YMC-2011. This prophage and YMC-2011 are more closely related to the *cos*-type phages than other *S. thermophilus*-infecting phages, suggesting that they originated from a common ancestor. The prophage from strain NCTC8618 and phage 5093 are in one cluster, suggesting that they share a common ancestor.

**Nature of the phage-specific genome.** To confirm that YMC-2011 exists as a circular plasmid, rather than as an integrated or linear prophage, the localization of YMC-2011 in *S. salivarius* 57.I was first determined by Southern hybridization, using YMC-2011-specific (an internal fragment of SsaI\_phage00042) and chromosome-specific (an internal fragment of *ureC*) probes. A YMC-2011-specific signal was observed in both the plasmid preparation and the total cellular DNA digested with NcoI (a unique cutter for YMC-2011), whereas the *ureC*-specific signals were detected only in NcoI-digested total cellular DNA, indicating that YMC-2011 exists extrachromosomally (Fig. 3A). To confirm that YMC-2011 is in a circular form, 9 pairs of primers were designed to generate overlapping amplicons around YMC-2011 (Fig. 1, fragments A through I). The amplicons were approximately 5 kbp and overlapped their flanking amplicons by at least 200 bp. All primer pairs were able to generate a PCR product of the expected length, confirming that YMC-2011 is a circular molecule (Fig. 3B). The copy number of YMC-2011 was then determined by quantitative real-time PCR (qPCR) with total DNA isolated from a stationary-phase culture (optical density at 600 nm [OD<sub>600</sub>] of 0.65) of *S. salivarius* 57.I. The YMC-2011 copy number in *S. salivarius* was estimated to be 3 or 4 copies per chromosome (Table 1), confirming that YMC-2011 exists as a circular plasmid.

**Induction and observation of phage particles.** We next wanted to examine phage particle production from YMC-2011. If a phage particle were to be produced upon mito-



**FIG 3** Analysis of the location and conformation of YMC-2011. (A) Southern hybridization with DIG-labeled DNA probes internal to *Ssal\_phage00042* (I) and *ureC* (II). The phage- and *ureC*-specific signals in panels I and II, respectively, are indicated by arrowheads. Lanes 1, uncut plasmid preparation; lanes 2, total cellular DNA digested with *NcoI*. (B) Overlapping PCR products (fragments A to I) amplified from plasmid YMC-2011. The relative locations of the amplicons are indicated in Fig. 1. M, 1-kb marker.

mycin C treatment, addition of SDS to destroy the capsid would be essential in order to obtain phage genomic DNA. To test this hypothesis, the assumed phage preparation was subjected to SDS treatment prior to PCR analysis. An *Ssal\_phage00002*-specific product, but not a *lacZ*-specific product, was obtained from the SDS-treated preparation, suggesting that mitomycin C treatment could induce phage production (Fig. 4A). An *Ssal\_phage00002*-specific PCR product, in lower abundance, was also observed in the sample without the SDS treatment, presumably resulting from phage particles damaged during the denaturation step of the PCR.

The purified phage preparation was negatively stained and examined by transmission electron microscopy (TEM). An icosahedral capsid with a long noncontractile tail, consistent with the *Siphoviridae* family, was observed (Fig. 4B). From four randomly selected images, the sizes for the icosahedral capsid and the tail were estimated to be  $60 \pm 1$  nm in diameter and  $232 \pm 1$  nm in length, respectively.

**Transcriptional organization of YMC-2011.** Based on the annotation, 49 ORFs of YMC-2011 are transcribed from one direction and 6 ORFs (*Ssal\_phage00010* to *Ssal\_phage00013*, *Ssal\_phage00016*, and *Ssal\_phage00043*) are transcribed from the opposite orientation (Fig. 1). Among those 6 ORFs, *Ssal\_phage00010* to *Ssal\_phage00013* are located in a cluster, and the products of the ORFs include an integrase (*Ssal\_phage00010*) and CI-like repressors (*Ssal\_phage00012* and *Ssal\_phage00013*) that

**TABLE 1** Estimation of the copy number of YMC-2011 by qPCR

Comparison	$\Delta C_q$ (mean $\pm$ SD) <sup>a</sup>	Copy number <sup>b</sup>
<i>ureC</i> - <i>Ssal</i> vs phage00002	$2.12 \pm 0.27$	
<i>codY</i> - <i>Ssal</i> vs phage00002	$2.29 \pm 0.16$	
<i>lacZ</i> - <i>Ssal</i> vs phage00002	$2.42 \pm 0.52$	
<i>ureC</i> - <i>Ssal</i> vs phage00031	$1.63 \pm 0.26$	
<i>codY</i> - <i>Ssal</i> vs phage00031	$2.19 \pm 0.15$	
<i>lacZ</i> - <i>Ssal</i> vs phage00031	$2.31 \pm 0.52$	
<i>ureC</i> - <i>Ssal</i> vs phage00063	$1.13 \pm 0.19$	
<i>codY</i> - <i>Ssal</i> vs phage00063	$1.61 \pm 0.35$	
<i>lacZ</i> - <i>Ssal</i> vs phage00063	$1.74 \pm 1.01$	
Mean $\pm$ SD <sup>c</sup>	$1.94 \pm 0.43$	3.84

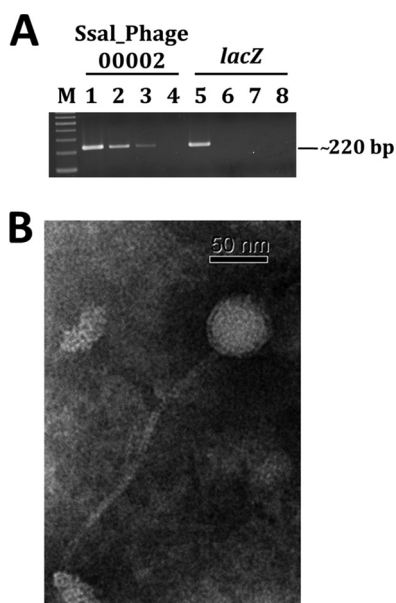
<sup>a</sup> $\Delta C_q$  is the  $C_q$  obtained from reactions specific for the chromosome (*ureC*, *codY*, and *lacZ*) minus the  $C_q$  obtained from reactions specific for YMC-2011 (*Ssal\_phage00002*, *Ssal\_phage00031*, and *Ssal\_phage00063*).

The values are the means  $\pm$  standard deviations (SDs) of three independent samples.

<sup>b</sup>The copy number was calculated as  $2^{\Delta C_q}$ .

<sup>c</sup>The values are the means  $\pm$  SDs of  $\Delta C_q$  values (mean values) obtained for all comparison pairs.

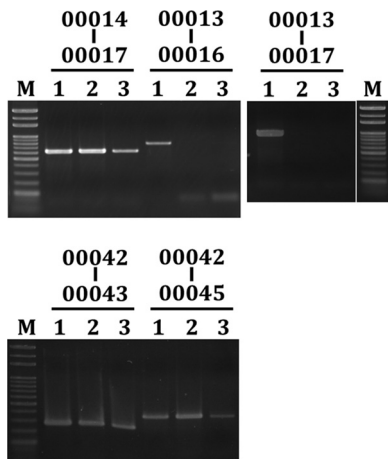




**FIG 4** Isolation and examination of *S. salivarius* 57.1 phage YMC-2011. (A) PCR products generated from the phage preparation. Primers specific for Ssal\_phage00002 of YMC-2011 (lanes 1 to 4) and the chromosome-borne *lacZ* (lanes 5 to 8) were used in PCRs with total cellular DNA (lanes 1 and 5), SDS-treated phage preparation (lanes 2 and 6), untreated phage preparation (lanes 3 and 7), or double-distilled water (lanes 4 and 8). M, 100-bp marker. (B) TEM of *S. salivarius* 57.1 phage YMC-2011. The phage was negatively stained with PTA.

may participate in lysogenic growth. Neither Ssal\_phage00016 nor Ssal\_phage00043 shares homology with any known sequences. The 49 ORFs that are transcribed in the opposite orientation encode proteins required for phage production. The arrangement of the YMC-2011 genome suggests that the juncture between Ssal\_phage00013 and Ssal\_phage00014 or that between Ssal\_phage00016 and Ssal\_phage00017, where two divergent transcripts are initiated, may contain one of the key *cis* elements controlling the switch between lysogenic growth and lytic growth. To test this hypothesis, reverse transcription (RT)-PCR was performed to analyze whether Ssal\_phage00014 to Ssal\_phage00064 and Ssal\_phage00001 to Ssal\_phage00009 were cotranscribed. The results revealed contiguous transcripts between Ssal\_phage00014 through Ssal\_phage00017 and Ssal\_phage00042 through Ssal\_phage00045 (Fig. 5). Expression in both regions was enhanced upon mitomycin C treatment, which is in agreement with the mitomycin C-induced phage production described above. In contrast, a contiguous transcript was not detected between Ssal\_phage00013 and Ssal\_phage00016 or Ssal\_phage00017, with or without mitomycin C treatment (Fig. 5), indicating that Ssal\_phage00013 and Ssal\_phage00016 are not cotranscribed and that a promoter is likely located 5' to Ssal\_phage00013. Using the same approach, we also investigated whether a contiguous transcript could be found between ORFs (in the same orientation), possessing an intergenic region of >150 bp; in all cases, a PCR product was obtained (data not shown). Taken together, the above observations suggest that at least two divergent transcripts, initiated from the 5'-flanking regions of Ssal\_phage00013 and Ssal\_phage00014, are transcribed from YMC-2011.

**Determination of transcription initiation sites of Ssal\_phage00013 and Ssal\_phage00014.** Given the results described above, we next determined, using 5' rapid amplification of cDNA ends (RACE), the transcription initiation sites for Ssal\_phage00013 and Ssal\_phage00014. Sequence analysis of the final PCR products mapped the transcription initiation site of Ssal\_phage00013 to an A located 26 bases 5' to the ATG (Fig. S3A). An extended  $-10$  sequence (5'-TGTGGTAATAT) was found to be located 6 bases 5' to the transcription initiation site (Fig. S3B). The transcription initiation site of Ssal\_phage00014 was mapped to an A located 22 bases 5' to the ATG (Fig. S3A), and



**FIG 5** Transcriptional organization of YMC-2011. RT-PCR was used to detect contiguous transcripts between two ORFs. Products for each of the intergenic regions are listed above the gel photograph. Lanes 1, PCR products generated from the total cellular DNA of *S. salivarius* 57.I; lanes 2 and 3, products generated from the cDNA of wild-type 57.I, with and without mitomycin C treatment, respectively. M, 1-kb marker.

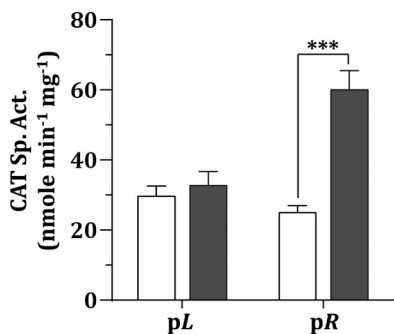
a typical  $\sigma^{70}$ -like promoter sequence (5'-TTGACA-N<sub>17</sub>-TATACT) was found 6 bases 5' to the transcription initiation site (Fig. S3B). Based on the relative locations of the two ORFs, the promoters for Ssal\_phage00013 and Ssal\_phage00014 were designated p<sub>L</sub> and p<sub>R</sub>, respectively.

The operator regulating the expression of lytic and lysogenic genes of a temperate phage often contains direct- or inverted-repeat sequences. Two imperfect inverted repeats were found in the intergenic region between Ssal\_phage00013 and Ssal\_phage00014. One (5'-ACTCAATTTCTGT) overlapped the extended -10 sequence of p<sub>L</sub>, and the other (5'-TACGAAAATTAGTA) was located between the -10 and -35 elements of p<sub>R</sub> (Fig. S3B). Based on the locations of the inverted repeats, it was suggested that they serve as targets for the antirepressor (encoded by Ssal\_phage00017) and the repressor (encoded by Ssal\_phage00012 and Ssal\_phage00013) of YMC-2011, respectively.

**Enhancement of p<sub>R</sub> transcriptional activity with mitomycin C treatment.** Because the expression of p<sub>R</sub> could potentially initiate lytic growth, whereas the expression of p<sub>L</sub> may be required for the maintenance of the lysogenic cycle, it was hypothesized that the expression of p<sub>R</sub> but not p<sub>L</sub> would be upregulated by mitomycin C treatment. To verify this hypothesis, the effects of mitomycin C treatment on the activity of p<sub>L</sub> and p<sub>R</sub> were examined using various chloramphenicol (Cm) acetyltransferase (*cat*) gene-reporter fusion strains. Mitomycin C treatment enhanced the activity of p<sub>R</sub> (2.4-fold increase), whereas it had no effect on the activity of p<sub>L</sub> (Fig. 6), suggesting that the expression of p<sub>R</sub> and p<sub>L</sub> is regulated, which determines the switch between lysogenic growth and lytic growth.

## DISCUSSION

The significant homology between the ORFs of YMC-2011 and ORFs from *S. thermophilus* phages suggests that YMC-2011 is associated with phage production. However, several approaches used to induce plaque formation in *S. salivarius* 57.I, including treatments with UV light and mitomycin C, failed to result in the observation of plaques or complete cell lysis. Nevertheless, phage particles were isolated from mitomycin C-induced *S. salivarius* 57.I cultures, indicating that a lysogenic stage of YMC-2011 has been established in strain 57.I. Furthermore, YMC-2011-specific PCR products were obtained from DNA isolated from phage particles (Fig. 4), confirming that the purified phages were generated from YMC-2011, rather than an unknown phage genome integrated into the chromosome. We also investigated, by PCR, whether YMC-2011 could exist as a phage particle in *S. salivarius*, in addition to a plasmid, without



**FIG 6** Expression analysis of the promoters of *Ssal*\_phage00013 ( $p_L$ ) and *Ssal*\_phage00014 ( $p_R$ ). The CAT specific activity of the  $p_L$ -*cat* and  $p_R$ -*cat* fusion strains was assayed after treatment with mitomycin C (■) or no treatment (□). Values are the means and standard deviations from three independent experiments. Significant differences between mitomycin C-treated samples and untreated samples were analyzed using Student's *t* test. \*\*\*,  $P < 0.001$ .

mitomycin C treatment. We found that DNase I treatment of total cell lysates of *S. salivarius* in the exponential growth phase abolished the phage-specific PCR product completely, and an additional SDS treatment did not result in the production of a phage-specific PCR product (data not shown), confirming that YMC-2011 is a "naked" virion without induction.

The expression of the bacteriophage holin-lysin system is essential for the degradation of host cell peptidoglycan and the development of a lytic cycle (30). When genes encoding the putative holin-lysin system of YMC-2011, *Ssal*\_phage00004, *Ssal*\_phage00006, and *Ssal*\_phage00007, were replaced with a nonpolar antibiotic resistance gene, we failed to isolate phage particles from mitomycin C-treated cultures using the standard procedure. However, phage particles were readily detected if the culture was lysed by mechanical disruption in a bead beater prior to phage isolation. Thus, the activity of the holin-lysin system is able to release phage particles but is ineffective in completely lysing host cells. *Ssal*\_phage00004 and *Ssal*\_phage00006 share 68% similarity, at the deduced amino acid level, with the holins (the products of *lyt49* and *lyt50*) of phage O1205 (31). A putative ribosome binding site (RBS) (5'-AGAGG) was found 6 bases 5' to the ATG translational start site of *Ssal*\_phage00004, but no RBS could be found 5' to *Ssal*\_phage00006. These two ORFs are 16 bases apart, and thus *Ssal*\_phage00006 may be translated via ribosome hopping, as has been seen for phage T4 (32). The translation of gene 60 of phage T4 utilizes programmed translational bypassing, in which two disparate ORFs, separated by a 50-nucleotide noncoding segment of mRNA, are translated into a single polypeptide. Further, studies have shown that only one-half of the bypassing events resume translation of the second ORF (33). Thus, it is possible that only a small amount of *Ssal*\_phage00006 is translated. However, a putative RBS (5'-GGAGG) is located 9 bases 5' to the ATG of *Ssal*\_phage00007, encoding a putative phage-associated cell wall hydrolase; therefore, even if the hydrolase is translated, the enzyme may cross the membrane ineffectively to hydrolyze the peptidoglycan of the host cell.

Phages generally infect host cells via specific receptors. For instance, phage DT1 utilizes the phage antireceptor (Orf18) to adsorb onto the surface of *S. thermophilus*, although the receptor on the bacteria has not yet been defined (34). *Ssal*\_phage00001 of YMC-2011 shares 75% similarity with Orf18 of DT1, suggesting that YMC-2011 is able to infect *S. thermophilus*. However, multiple attempts to detect any plaques in *S. thermophilus* ATCC BAA-250 (35) and *S. salivarius* ATCC 25975 (36) infected with purified YMC-2011 phage particles failed. This observation has three possible explanations, i.e., (i) *Ssal*\_phage00001 may not be responsible for infection, (ii) the expression of  $p_R$  may not be induced in these two strains, or (iii) the holin-lysin system may not be active enough to lyse the cell walls, as seen with *S. salivarius* 57.1.

A lysogenic state can be maintained with either an integrated prophage or a plasmid with a low copy number. In general, prophages result from integration of the



phage genome into the host chromosome at a specific site through the activity of a phage integrase. For instance, a 40-bp region, known as *attP*, in O1205 (37) and Sfi21 (38) is the site of recombination between the phage and the host chromosome. This region is quite conserved, as the prophage of *S. salivarius* strain JIM8777 is also integrated at this site. While this core sequence is found in YMC-2011 and the *S. salivarius* 57.1 chromosome, at locations analogous to those seen in *S. thermophilus* and *S. salivarius* JIM8777, plasmid YMC-2011 could also exist as an integrated prophage, as seen in strain JIM8777. When we examined, by PCR, the distance between ORFs flanking the putative *attB* locus in *S. salivarius* 57.1, we obtained an amplicon whose size was indicative of a genome-derived origin (data not shown), confirming that YMC-2011 is not integrated, at least not at the putative *attB* locus. When we compared the putative integrase (Ssal\_phage00010) of YMC-2011 with the integrases of phages O1205 and Sfi21 and the prophage of *S. salivarius* JIM8777, we found that, first, Ssal\_phage00010 shared the least sequence identity at the deduced amino acid level with the other three integrases and, second, most of the variations were located at the N terminus. Based on the analysis of the conserved domains, this region is proposed to harbor an AP2-like DNA-binding integrase domain. Thus, it is possible that YMC-2011 lacks an active integrase.

In contrast to phages isolated from *L. lactis*, which consist of at least 10 genetically distinct groups (39), the phages from *S. thermophilus* are derived from a single ancestor (11); thus, YMC-2011 is likely to be derived from the same ancestor, based on the sequence homology between YMC-2011 and *S. thermophilus* phages. Comparative sequence analysis revealed that two distinct processes, i.e., recombination and the accumulation of point mutations, created diversity among *S. thermophilus* phages. Recombination is apparently the basis for observed differences in lifestyle (virulent versus temperate phages), host range, and DNA packaging systems, whereas point mutations contribute approximately 10% of the sequence diversity observed among *S. thermophilus* phages (40). It is possible that the observed divergence between YMC-2011 and the known *cos*-type phages may be derived from both recombination events and point mutations.

The mechanisms for the lytic-lysogenic switch are quite similar among temperate phages. Similar to  $\lambda$ , two promoters, responsible for lytic and lysogenic growth, are arranged adjacently in opposite orientations in several Gram-positive bacteria phages, including Tuc2009, TP901-1, BK5-T, r1t, and  $\phi$ LC3 of *L. lactis* and Sfi21 of *S. thermophilus* (41), although only two operators, instead of three in  $\lambda$ , with palindromic sequences are observed between  $p_L$  and  $p_R$  in all of those phages. The repressor and the antirepressor compete for overlapping operator sites, which regulate the switch between the lysogenic state and the lytic state in most temperate lactic acid phages (42). Furthermore, the two CI repressor proteins of YMC-2011 share 52% and 81% identity with ORF127 and ORF122 of Sfi21, respectively, suggesting that the sequence of the operator in YMC-2011 is similar to that in Sfi21. Therefore, it is likely that the expression of  $p_L$  and  $p_R$  of YMC-2011 determines the switch between the lysogenic stage and the lytic stage, as seen in Sfi21.

In conclusion, we demonstrated that YMC-2011 could generate phage particles upon mitomycin C induction. The presence of YMC-2011 may provide immunity for *S. salivarius* 57.1 against infection with related phages.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this work are listed in Table 2. *S. salivarius* 57.1 was cultivated in brain heart infusion (BHI) medium (Difco) at 37°C in a 10% CO<sub>2</sub> atmosphere. Recombinant streptococcal strains were maintained in BHI medium containing spectinomycin (Sp) at 750  $\mu$ g ml<sup>-1</sup>, erythromycin (Em) at 5  $\mu$ g ml<sup>-1</sup>, or kanamycin (Km) at 800  $\mu$ g ml<sup>-1</sup>, as needed. Recombinant *Escherichia coli* strains were cultured in LB broth containing ampicillin (Ap) at 100  $\mu$ g ml<sup>-1</sup> or Sp at 100  $\mu$ g ml<sup>-1</sup>, as needed.

**In silico analysis of YMC-2011.** The complete sequence of YMC-2011 (GenBank accession number CP002889.1) was obtained from GenBank. The basic characteristics of ORFs of  $\geq 30$  amino acids in YMC-2011 were analyzed using Vector NTI Advance 11 (Invitrogen). The annotation of the ORFs was verified using BLASTX (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A DnaA box consensus sequence, derived

**TABLE 2** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype <sup>a</sup>	Description	Source
<i>S. salivarius</i> strains			
57.1		Wild-type strain	59
$\Delta codY$	Em <sup>r</sup> , CodY <sup>-</sup>	Strain 57.1 <i>codY::erm</i>	52
$\Delta codY_{p_L-cat}$	Em <sup>r</sup> , Sp <sup>r</sup> , CodY <sup>-</sup>	Strain $\Delta codY$ harboring <i>spe-p_L-cat</i> fusion at <i>lacZ</i>	This study
$\Delta codY_{p_R-cat}$	Em <sup>r</sup> , Sp <sup>r</sup> , CodY <sup>-</sup>	Strain $\Delta codY$ harboring <i>spe-p_R-cat</i> fusion at <i>lacZ</i>	This study
CodY+/ $\Delta codY_{p_L-cat}$	Sp <sup>r</sup> , Km <sup>r</sup> , Em <sup>s</sup> , CodY <sup>+</sup>	<i>codY::erm</i> in strain $\Delta codY_{p_L-cat}$ replaced by $\Omega kan$ -tagged <i>codY</i>	This study
CodY+/ $\Delta codY_{p_R-cat}$	Sp <sup>r</sup> , Km <sup>r</sup> , Em <sup>s</sup> , CodY <sup>+</sup>	<i>codY::erm</i> in strain $\Delta codY_{p_R-cat}$ replaced by $\Omega kan$ -tagged <i>codY</i>	This study
Plasmid			
pMC300	Ap <sup>r</sup> , Sp <sup>r</sup>	Integration vector for <i>S. salivarius</i> harboring <i>p_urei-cat</i> fusion	52

<sup>a</sup>r, resistant; s, sensitive.

from the putative DnaA box sequences of *S. salivarius* strains in DoriC (<http://tubic.tju.edu.cn/doric/search1.php>) (43, 44), was used to search for the putative *ori* of YMC-2011 using Ori-Finder (45). Sequence alignment was analyzed using ClustalW at the San Diego Supercomputer Center Biology Workbench (<http://workbench.sdsc.edu>).

The whole-genome sequences of phages O1205 (GenBank accession number [U88974.1](#)), 858 (GenBank accession number [EF529515.1](#)), ALQ13.2 (GenBank accession number [FJ226752.1](#)), Abc2 (GenBank accession number [NC\\_013645.1](#)), DT1 (GenBank accession number [AF085222.2](#)), Sfi19 (GenBank accession number [AF115102.1](#)), Sfi21 (GenBank accession number [AF115103.1](#)), 2972 (GenBank accession number [NC\\_007019.1](#)), Sfi11 (GenBank accession number [NC\\_002214.1](#)), 7201 (GenBank accession number [NC\\_002185.1](#)), 9871 (GenBank accession number [KU678389](#)), 9872 (GenBank accession number [KU678390](#)), 9873 (GenBank accession number [KU678391](#)), 9874 (GenBank accession number [KU678392](#)), and 5093 (GenBank accession number [NC\\_012753.1](#)) of *S. thermophilus* were obtained from GenBank. The sequences of the putative prophages of *S. salivarius* were extracted from the genomes of *S. salivarius* strains JF (GenBank accession number [NZ\\_CP014144.1](#)), NCTC8618 (GenBank accession number [NZ\\_CP009913.1](#)), and JIM8777 (GenBank accession number [NC\\_017595.1](#)), respectively. The evolutionary relationships of the aforementioned phages and prophages and YMC-2011 were analyzed using MEGA 6.0 (46).

**Southern hybridization.** Total cellular DNA (47) and plasmid DNA (48) were isolated from *S. salivarius* 57.1 as described previously. Purified DNA was separated on a 0.5% agarose gel and subjected to Southern hybridization analysis. Probes specific for chromosome-borne *ureC* and Ssal\_phage00042 of YMC-2011 were generated by PCR using the primer pairs *ureC\_p\_S/ureC\_p\_AS* and *phage\_24328\_S/phage\_25698\_AS*, respectively. All primers used in this study are listed in Table 3. The PCR products were purified and then labeled with digoxigenin (DIG) using a DIG DNA labeling and detection kit (Roche). Hybridization was carried out at 52°C for 16 h in hybridization buffer (4× Denhardt's solution, 3× SSC [standard saline citrate] [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% SDS, 100 μg ml<sup>-1</sup> salmon sperm DNA). The unbound probes were removed with two 5-min washes at 30°C at low stringency (1× SSC, 0.1% SDS), followed by two 15-min washes at 50°C at high stringency (0.1× SSC, 0.1% SDS). The bound probe was detected using the DIG detection kit, according to the manufacturer's instruction (Roche).

**Confirmation of the circular structure and determination of the copy number of YMC-2011.** The conformation of YMC-2011 as a circular molecule was verified by PCR with primers generating overlapping fragments around YMC-2011. The copy number of YMC-2011 was determined by real-time qPCR, based on the method described by Chen et al. (49), with minor modifications. The reactions were conducted using iQ SYBR green supermix (Bio-Rad) and a 7500 fast real-time PCR system (Applied Biosystems). Primers specific for Ssal\_phage00002 (*phage\_3019\_S\_002* and *phage\_3245\_AS\_002*), Ssal\_phage00031 (*phage\_18928\_S\_031* and *phage\_19142\_AS\_031*), and Ssal\_phage00063 (*phage\_36884\_S\_063* and *phage\_37122\_AS\_063*) from YMC-2011 and for the chromosomal genes *lacZ* (*lacZ\_1241\_S* and *lacZ\_1465\_AS*), *codY* (*codY\_1170\_S* and *codY\_1280\_AS*), and *ureC* (*ureC\_6301\_S* and *ureC\_6460\_AS*) were designed and used for qPCR. All primers were designed to have a predicted melting temperature of ~55°C and a product of approximately 250 bp. The copy numbers were calculated as the mean quantification cycle ( $C_q$ ) values of the chromosomal genes, compared to the phage-specific genes, using the formula  $2^{\Delta C_q}$ , where  $\Delta C_q$  is the  $C_q$  obtained with primers specific for chromosome-borne genes minus the  $C_q$  obtained with primers specific for the ORFs of YMC-2011.

**Induction and examination of phage particles.** An early-exponential-phase culture (an OD<sub>600</sub> of 0.25 to 0.3) of *S. salivarius* 57.1 in BHI medium was treated for 3 h with mitomycin C (Sigma) at a final concentration of 0.625 μg ml<sup>-1</sup>. At the end of the treatment, the culture was subjected to centrifugation at 3,500 rpm for 10 min at 4°C, and the supernatant was recovered. Cell debris in the supernatant was removed by filtration through a 0.45-μm-pore-size membrane (Pall). Phage particles in the supernatants

**TABLE 3** Primers used in this study

Primer	Sequence <sup>a</sup>	Purpose	
ureC_p_S	GACATCAAAGGTCTGTGGATCG	Generation of probes used in Southern analysis	
ureC_p_AS	CTGGTCTGTGCTGATGGAAGTAA		
phage_24328_S	GAGCCGCCACAAGGTGTTCTTT	Conformation of YMC-2011 as circular molecule by PCR	
phage_25698_AS	CGCACTCACATAACCACCTCC		
phage_1_S	TGGCATGTTGCCATACTCAAACAGT		
phage_5000_AS	CTGTAGTTCTTCTTCTACGACGTT		
phage_4776_S	ACACAAAGAATGCGCAGTGTGGCAT		
phage_9776_AS	ATGTCATTTATTGGGGATATCACCG		
phage_9531_S	CACAGATAGAGACATTGAACTGCTC		
phage_14550_AS	TGTTCCCAATCGATTGAATCCAAGG		
phage_14326_S	TTGACGGCTCGGACTTTGAACGCAC		
phage_19390_AS	TGTAATCGTCTTTGATTGCCCAATT		
phage_19140_S	GTACAAGCTGGTATCTACGTGGATG		
phage_24170_AS	GTAGCTCGGTGTTCTGTTTCGTTCT		
phage_23871_S	ACCGAGATGATGAGAGTAAGGAACG		
phage_29000_AS	TACCATTGGCATTAGGGCGTTCCT		
phage_28800_S	CGTCACTTTTGATGATCCACGCATT		
phage_33865_AS	AGAGATTTCTGTGTGCGGACATTG		
phage_33651_S	CAAGCAACCCTAATGTGTTGCGTAT		
phage_38666_AS	AACCGTTACGGATATTGTTGTGCC		
phage_38431_S	CGATGGCGTGATTCCGGTTGTTGAG		
phage_2600_AS	AGCGCTCTGACATATCGCTCAGATT		
phage_3019_S_002	GGTTACGTCGATGGTTTTGGGC	qPCR for YMC-2011 copy number estimation	
phage_3245_AS_002	CCTCTTGGGATAGTTGTCAGTG		
phage_18928_S_031	TGGCAAGTCATTTCTGCCAGCC		
phage_19142_AS_031	ACATCGTCACCAATAATGACGG		
phage_36884_S_063	GGGATAACGTC AAGAAATTCGC		
phage_37122_AS_063	GGTCAATGCGTCGGTGATTAGC		
codY_1170_S	GGCTGCTCAATTGGCGGATATCAT		
codY_1280_AS	CTCCTCCACACGATCATTGTTGGTT		
lacZ_1241_S	GGGATATCAAAGTGATGAAAC		
lacZ_1465_AS	GGCACGATCCAACAAGC		
ureC_6301_S	GGACCAACTGTAGGTGATAGCGTAC	Negative control for Fig. 4	
ureC_6460_AS	TGGATTGTCACGTGTTCCGTAGC		
lacZ_1241_S	GGGATATCAAAGTGATGAAAC		
lacZ_1465_AS	GGCACGATCCAACAAGC		
phage_9787_013_S	ATTCTCAGCTTTTGCCTTCATG		RT-PCR for transcription organization study
phage_10755_017_AS	ACTGATTACTGGCTCATGATTT		
phage_10134_014_S	CAGACTTGCTCGGAGTTGATT		5' RACE for Ssal_phage00013
phage_20582_016_AS	CAACGAATCTGTTAGCGAAGC		
phage_24765_042_S	AGCCATGCAACTAGGTCTCA		
phage_25034_043_AS	ATGGCAAAAAACGTATGACGT		
phage_25162_045_AS	TGTGCTTCCGTCTGCTCAT		
phage_10757_R1_AS	GCACTGATTACTGGCTCATG		
phage_10653_R2_AS	GGAGAGACAAGCCATGATTGAA		
phage_10514_R3_AS	TTGCTATTTCAAGTGCTATCGC		
phage_9251_L1_S	CTCATCACAATCTGTTGTTGAG	5' RACE for Ssal_phage00014	
phage_9369_L2_S	TTTCACGCAACCGTTCATAGTC		
phage_9479_L3_S	CTGTTTACAGGGTCGATAAAGC	Construction of p <sub>L</sub> -cat and p <sub>R</sub> -cat fusions	
lacZ_S	GGTTTTGGTTCTCCACAATATGTG		
purel_481SmaI_AS	AATCCCGGGGACCATATGGGAGTCCGTAC		
purel_958Sall_S	GAGGTCGACATGAACTTTAATAAAATTGATTT		
lacZ_AS	CAGCAACATATTGACCGCGAAC		
phage_9943SmaI_PR_S	GTACCCGGGATTTCTCTTTCTTCTATATAT		
phage_10067Sall_PR_AS	ATTGTCGACCTCCTTCTTAAGCTTGATTTAAGTA		
phage_10067SmaI_PL_S	GCTCCCGGGTTCCTTAAGCTTGATTTAAGTA		
phage_9943Sall_PL_AS	TTTGTGACCTCCATTTCTCTTTCTTCTATATAT		
codY+_815BamHI_S	TTGGATCCCTGGACAAAAAGCCTTGTC		PCR for establishing intact <i>codY</i> in strain $\Delta$ <i>codY</i>
codY+_3606SphI_AS	ATAGCATGCCTTGACATTCTTTGAAGAGG		
codY+_518SacI_S_1	GGTGAGCTCGTTTCAGAGATGATTTCCATGT		
codY+_824BamHI_AS	AGGGATCCAAGAAATTAACGCTTGTAAATATG		

<sup>a</sup>Inserted restriction recognition sites are underlined.

were collected by ultracentrifugation at 25,000 rpm for 2 h at 4°C. The purified phage particles were suspended in 10 mM Tris-HCl (pH 8) and stored at 4°C until use. An aliquot of the phage suspension (5 μl) was deposited on top of a Formvar/carbon-coated 200-mesh grid, and the grid was set at room temperature for 1 min. Excess liquid on the grid was removed with a piece of Whatman paper. The grid

was then negatively stained for 1 min with 2% phosphotungstic acid (PTA) (pH 7). The staining solution was removed with a piece of Whatman paper. The grid was dried in a chamber under low vacuum pressure. The morphology of the phage particles was observed using a JEOL 1230 transmission electron microscope, at a magnification of  $\times 200,000$ . The size of phage YMC-2011 was estimated using AxioVision LE (Zeiss).

**Isolation of YMC-2011 phage DNA.** DNA was isolated from the phage particles by the method described by Zinno et al. (50), with minor modifications. Briefly, the phage preparation was treated initially with DNase I and RNase A to remove free DNA and RNA in the preparation. The resulting suspension was then treated at 65°C for 30 min with 100  $\mu$ l of 2.5% SDS in 0.5 M Tris-HCl, 0.25 M EDTA (pH 8), to remove the protein coat. The released DNA was further purified by phenol-chloroform (1:1) extraction and ethanol precipitation. The purity of the phage DNA was verified by PCR with two primer pairs, i.e., phage\_3019\_S\_002/phage\_3245\_AS\_002 (specific for Ssa1\_phage00002) and lacZ\_1241\_S/lacZ\_1465\_AS (specific for chromosome-borne *lacZ*).

**RNA isolation, RT-PCR, and 5' RACE.** Total cellular RNA was isolated from *S. salivarius* 57.I by the method described by Chen et al. (51). The residual DNA in the RNA preparation was removed with DNase I. The final product was further purified using an RNeasy minikit (Qiagen). To investigate the transcriptional organization of YMC-2011 by RT-PCR, 2  $\mu$ g of purified RNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase and random primers (Promega). The cDNA was used in the PCR with primers specific for YMC-2011.

The transcriptional start sites of Ssa1\_phage00013 and Ssa1\_phage00014 from YMC-2011 were determined using the 5' RACE system (Invitrogen); 5  $\mu$ g of total RNA from *S. salivarius* 57.I was used in the 5' RACE reaction. Ssa1\_phage00013- and Ssa1\_phage00014-specific cDNAs were synthesized from the total RNA using primers phage\_9251\_L1\_S and phage\_10757\_R1\_AS, respectively. After removal of the mRNA template and addition of the poly(C) tail to the cDNA pieces, the abridged anchor primer (AAP) was paired with primers phage\_9369\_L2\_S and phage\_10653\_R2\_AS to amplify Ssa1\_phage00013- and Ssa1\_phage00014-specific cDNA, respectively; 0.1% of the final products was then reamplified by nested PCR using the abridged universal amplification primer (AUAP) paired with the primers phage\_9479\_L3\_S and phage\_10514\_R3\_AS, respectively. Notably, the AUAP contains an adapter region that is complementary to the adapter region of the AAP. The final PCR products were separated by agarose gel electrophoresis and purified for sequencing analysis.

**Construction of recombinant *S. salivarius* strains.** *S. salivarius* 57.I is not naturally competent; therefore, it is rather difficult to generate recombinant strains. Our recent studies revealed that a spontaneous mutation had occurred in the 5'-flanking region of *comX* in a *codY*-deficient derivative of *S. salivarius* 57.I (strain  $\Delta codY$ ) (52). The mutation resulted in enhanced *comX* expression and a naturally competent phenotype (unpublished results by Y.-Y. Kao and Y.-S. Yen). Thus, *S. salivarius*  $\Delta codY$  was used to generate recombinant strains harboring a Ssa1\_phage00013 promoter ( $p_L$ -*cat* fusion and a Ssa1\_phage00014 promoter ( $p_R$ -*cat* fusion in the *lacZ* locus, by using PCR ligation mutagenesis (53). Briefly, three DNA fragments were prepared by PCR. First, the intergenic region between Ssa1\_phage00013 and Ssa1\_phage00014 was amplified from *S. salivarius* 57.I with two sets of primers, i.e., phage\_99433SmaI\_PR\_S/phage\_10067SmaI\_PR\_AS and phage\_10067SmaI\_PL\_S/phage\_99433SmaI\_PL\_AS. The second DNA fragment, containing the 5' portion of *lacZ* fused to a *Sp* resistance gene (*spe*), was amplified from pMC300 (52) with the primers lacZ\_S and pureI\_481SmaI\_AS. The third fragment, containing a promoterless *cat* (54) fused with the 3' portion of *lacZ*, was amplified from pMC300 with the primers pureI\_958SmaI\_S and lacZ\_AS. Notably, a RBS (5'-GGAGG) was located 5 bases 5' to the translational start site of *cat*. Restriction sites were included in the primers to facilitate cloning. All PCR products were digested with SmaI and/or SmaI to allow for ligation in the following order: *lacZ-spe* followed by  $p_L$ -*cat*/ $p_R$ -*cat* and the 3' portion of *lacZ*. The resulting ligation mixture was introduced into *S. salivarius*  $\Delta codY$  by natural transformation (55), and the correct recombination event in the *Sp*-resistant transformants was verified by colony PCR using *lacZ*-specific primers. The recombinant strains harboring the  $p_L$ -*cat* and  $p_R$ -*cat* transcriptional fusions in the *lacZ* locus were designated  $\Delta codY$ - $p_L$ -*cat* and  $\Delta codY$ - $p_R$ -*cat*, respectively. Once the desired constructs were established in strain  $\Delta codY$ , an intact *codY* was reestablished in the host using a similar approach. To accomplish this, a 2-kbp amplicon containing the promoter and coding sequence of *codY* and its 3'-flanking region of 550 bp was amplified from *S. salivarius* 57.I with primers codY+\_815BamHI\_S and codY+\_3606\_SphI\_AS. An amplicon containing the 5'-flanking gene of *codY*, *alaA*, was generated from *S. salivarius* 57.I by using primers codY+\_518SmaI\_S\_1 and codY+\_824BamHI\_AS. Both PCR products were digested with BamHI and then ligated to the  $\Omega kan$  cassette (56) on a BamHI fragment. The resulting ligation mixture was used to transform strains  $\Delta codY$ - $p_L$ -*cat* and  $\Delta codY$ - $p_R$ -*cat*, and transformants were selected on agar containing Km. The correct recombination event was verified by colony PCR using *codY*-specific primers. The resulting recombinant strains were designated CodY+/ $\Delta codY$ - $p_L$ -*cat* and CodY+/ $\Delta codY$ - $p_R$ -*cat*, respectively.

**CAT assay.** Recombinant *S. salivarius*  $p_L$ -*cat* and  $p_R$ -*cat* fusion strains were cultivated in BHI medium to an OD<sub>600</sub> of 0.25 to 0.3, followed by a 1-hour treatment with mitomycin C; control cultures were grown and not treated. At the end of the treatment, the cultures were harvested, washed once with 10 mM Tris-HCl (pH 7.8), and resuspended in the same buffer in 2.5% of the original culture volume. Total lysates were prepared as described previously (57). The protein concentrations of the lysates were measured using the Bio-Rad protein assay. CAT activities were determined by the method described by Shaw (58), and specific activity values were calculated as nanomoles of Cm acetylated per minute per milligram of total protein. All reactions were performed in triplicate, and negative-control reactions were performed in the absence of the substrates. Statistical analysis between mitomycin C-treated and untreated samples was performed using the unpaired two-tailed Student's *t* test.



## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03186-16>.

**TEXT S1**, PDF file, 0.5 MB.

## ACKNOWLEDGMENTS

This work was supported by the Ministry of Science and Technology, Republic of China (grant NSC102-2320-B-182\_031-MY3 to Y.-Y.M.C.).

We thank R. Faustoferri and S. T. Liu for review of the manuscript.

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