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1 **Generation of bacteriophage-insensitive mutants of *Streptococcus thermophilus* using an**
2 **antisense RNA CRISPR-Cas silencing approach.**

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19 Running title: *S. thermophilus* BIM generation by CRISPR-Cas silencing.

20 Keywords: bacteriophage, resistance, insensitive, mutant, mRNA, adsorption, receptor,
21 sedimentation.

22 **Abstract**

23 Predation of starter lactic acid bacteria such as *Streptococcus thermophilus* by bacteriophages
24 is a persistent and costly problem in the dairy industry. CRISPR-mediated Bacteriophage
25 Insensitive Mutants (BIMs), while straightforward to generate and verify, can quickly be
26 overcome by mutant phages. The aim of this study was to develop a tool allowing the
27 generation of derivatives of commercial *S. thermophilus* strains which are resistant to phage
28 attack through a non-CRISPR-mediated mechanism, with the objective of generating BIMs
29 exhibiting stable resistance against a range of isolated lytic *S. thermophilus* phages. To
30 achieve this, standard BIM generation was complemented by the use of the wild-type (WT)
31 strain which had been transformed with an antisense mRNA-generating plasmid (targeting a
32 crucial CRISPR-associated [*cas*] gene), in order to facilitate the generation of non-CRISPR-
33 mediated BIMs. Phage sensitivity assays suggest that non-CRISPR-mediated BIMs exhibit
34 some advantages compared to CRISPR-mediated BIMs derived from the same strain.

35

36 **Importance:** The outlined approach reveals the presence of a powerful host-imposed barrier
37 for phage infection in *S. thermophilus*. Considering the detrimental economic consequences
38 of phage infection in the dairy processing environment, the developed methodology has
39 widespread applications, particularly where other methods may not be practical or effective
40 in obtaining robust, phage-tolerant *S. thermophilus* starter strains.

41 **Introduction**

42 *Streptococcus thermophilus* is a widely employed bacterium used for milk acidification and
43 subsequent product texturizing, required for the manufacture of cheeses and yoghurts in the
44 dairy industry (1). This process, however, can be disrupted, retarded or halted by contaminant
45 (bacterio)phages, which present a persistent problem in dairy fermentations (2, and reviewed
46 by 3). Several approaches may be utilised in an attempt to mitigate this costly problem. These
47 include: (i) starter rotational schemes (i.e. the rotation of strain sets with non-overlapping
48 phage sensitivities), (ii) heat or chemical treatments of dairy plant equipment in order to
49 reduce the phage burden (4), or (iii) generation of bacteriophage insensitive mutants (BIMs)
50 of *S. thermophilus* starters for the incorporation into rotational schemes mentioned above.
51 BIMs may be generated by one of several methods, including insertional mutagenesis (5),
52 immunoselection combined with cell sorting (6), construction of mutant libraries followed by
53 phage exposure (7), the secondary culture method (8), serial passaging in the presence of high
54 titer phages (9, 10), or chemical mutagenesis (11-13)

55 Generated BIMs should, ideally, be (i) robust, i.e. insensitive to a high level of and/or
56 repeatedly introduced phages, (ii) resistant to a wide variety of genetically distinct phages,
57 (iii) possess identical technological properties to the parent strain from which they were
58 derived, and (iv) be derived from parent *S. thermophilus* strains at a high frequency, and (v)
59 easy to characterise both genetically and phenotypically. In the context of robustness, defined
60 here as the ability of a BIM to survive high titer phage exposure, repeated phage challenges
61 (such as the Heap-Lawrence test (14), and/or challenges using genetically distinct phages, the
62 mechanism conferring resistance to the BIM may be relevant. Several *S. thermophilus* phage
63 resistance systems have been documented. Restriction modification (R/M) systems are
64 thought to be widespread in this species and are, for the most part, chromosomally encoded
65 (15-19), with one described example of a complete, plasmid-encoded R/M system (20). A

66 lactococcal-derived R/M system was also shown to function in *S. thermophilus* when
67 introduced using a compatible cloning vector (21). A lactococcal abortive infection (Abi)
68 system was shown to function in *S. thermophilus* (22), and indeed, evidence of a native Abi
69 system has been detected (23), though these systems are not well characterised in *S.*
70 *thermophilus*. Superinfection exclusion (Sie) has also been described in prophages of *S.*
71 *thermophilus* (24), although, considering the apparent rarity of resident prophages in the
72 species (24, 25), prophage-encoded systems of this type are probably not widespread.

73 The most widely characterised phage-resistance system in *S. thermophilus* is the
74 clustered regularly interspaced short palindromic repeats (CRISPR), and their associated
75 ‘CRISPR-associated’, or *cas*, genes - together referred to as the ‘CRISPR-Cas system’ (26-
76 29), first proven to be a phage-resistance mechanism by Barrangou and colleagues (30). Four
77 distinct CRISPR-Cas systems have now been identified in *S. thermophilus* (31), of which two
78 are known to actively provide phage immunity to this species (this study, 26, 27) - namely
79 CRISPR1-Cas and CRISPR3-Cas. Both of these systems are classified as ‘Type II-A’
80 systems, of which the *cas* components comprise *cas9*, *cas1*, *cas2* and *csn2* - each performing
81 a specific function in the context of the system (32).

82 These systems fundamentally act to digest incoming DNA using the product of the *cas9*
83 gene the signature gene of the Type II-A group (33, 34)), which is essential for CRISPR-
84 based immunity (30, 35, 36). Segments (approximately 30 base pairs) of the foreign DNA
85 (known as ‘spacers’) are incorporated into the chromosome at specific repeat-spacer loci in a
86 mechanism which is believed to involve the product of the *csn2* gene (30, 37), potentially in
87 complex with Cas9, Cas1, and Cas2 (38). The Cas-encoding genes, as well as the repeat-
88 spacer loci are constitutively transcribed (18, 39), and the resultant ‘crRNA’ acts as a guide to
89 particular Cas proteins to recognise and target subsequent incoming DNA. This process is
90 reliant on the presence of an approximately 30 nucleotide DNA fragment on the phage

91 genome termed the protospacer (i.e., the specific sequence on the phage genome that is
92 targeted by the CRISPR-Cas system; 40) as well as a short sequence flanking this target
93 sequence, termed the protospacer adjacent motif or PAM (26, 40, 41).

94 The presence of up to four distinct CRISPR-Cas systems in characterised *S.*
95 *thermophilus* strains (31) suggests that this system has been instrumental in shaping the
96 dynamics of the phage-host relationship on an evolutionary scale. The efficiency and
97 specificity of this immune system is matched only by the phages' adaptive response to it.
98 Deveau and colleagues (40) showed that phages can rapidly overcome CRISPR-mediated
99 resistance through mutations in either the target protospacer or adjacent PAM. Similar results
100 were reported more recently by Sun and colleagues (42). Thus, in order to develop robust
101 BIMs, it may be prudent to maximise the number of systems targeting the incoming phage,
102 an approach which has recently been shown to significantly increase phage resistance by
103 combining the protective effect of a CRISPR-Cas system with that of a functional R/M
104 system (43). In order to facilitate the selection of non-CRISPR-mediated BIMs, we
105 endeavoured to inactivate (by mRNA silencing) purportedly crucial genes in the CRISPR1-
106 Cas and CRISPR3-Cas systems, namely *cas9* and *csn2*.

107 Here we present a method whereby BIMs of *S. thermophilus*, whose phage resistance is
108 mediated by a CRISPR-independent mechanism, may be selected for. This method relies on
109 antisense mRNA-producing plasmids, a method previously used to confer phage resistance to
110 *Lactococcus lactis* and *S. thermophilus* by targeting intracellular phage replication (44-47).
111 Here, the principle of anti-sense-mediated 'CRISPR-Cas silencing' was validated by
112 targeting the *cas9-1* (CRISPR1-associated) or *cas9-3* (CRISPR3-associated) genes of three
113 BIMs, thereby inhibiting phage resistance conferred by a previously acquired spacer. In order
114 to generate BIMs of a given *S. thermophilus* strain that are resistant to phages by the action of
115 a non-CRISPR-mediated mechanism, an anti-sense plasmid construct simultaneously

116 silencing both the *csn2* gene of the CRISPR1-Cas system and its analogue in the CRISPR3-

117 Cas system was employed.

118

119 **Materials and methods**

120 *Isolation, growth and storage of bacterial strains and bacteriophages*

121 Bacterial strains and bacteriophages applied in this study are listed in Table 1. *S.*
122 *thermophilus* strains were routinely grown from 20 % Reconstituted Skimmed Milk (RSM)
123 stocks or from a single colony overnight at 42 °C in M17 Broth (Oxoid, Hampshire, U.K.)
124 supplemented with 0.5 % lactose (LM17) or on plates containing 10 g/L technical agar
125 (Merck, Darmstadt, Germany). Phages were isolated from industrial whey samples and
126 purified by two rounds of single plaque purification as previously described (48). Phage
127 enumeration assays were performed as previously described (49) using LM17 broth
128 supplemented with 0.25 % glycine (Oxoid, U.K.), 10 mM CaCl₂ (Oxoid, U.K.) and either 10
129 g/L (solid agar base) or 4 g/L (semi-solid overlay) technical agar. *L. lactis* NZ9000 (50) was
130 maintained as above with the following modifications: overnight (ON) cultures were grown
131 at 30 °C with the substitution of glucose (Sigma-Aldrich, St. Louis, MO, U.S.A.) instead of
132 lactose. All transformants were maintained as above with the addition of chloramphenicol
133 (Cm; Sigma-Aldrich) to a final concentration of 5 µg/ml (L or GM17+Cm5).

134

135 *Bacteriophage Insensitive Mutant (BIM) generation and validation*

136 BIMs of *S. thermophilus* ST47795 (hereafter referred to as strain ‘STA’) and ST64985
137 (hereafter referred to as strain ‘STB’) were generated by adding 300 µl fresh overnight
138 bacterial culture and 10 µl undiluted phage lysate (at a titer of approximately 1 x 10⁷ pfu/ml)
139 to 4 ml of semi-solid agar (as above), in a modification as outlined in a previously published
140 plaque assay method (49). Colonies visibly growing in the semi-solid agar following
141 overnight incubation (where the bacterial lawn failed to grow due to the presence of phages)
142 were twice single-colony purified and subjected to phage challenge (as described above), and
143 selected for further characterisation (as described below). In cases where an insufficient

144 number of BIMs were isolated using this method, the amount of culture and/or phage lysate
145 added was increased, or the method repeated. Sensitivity of wild-type and derived BIMs to
146 phages was determined using spot and/or plaque assays, as previously described (49). The
147 relative efficiency of plaquing (EOP) of phages on wild-type strains and derived BIMs was
148 determined by dividing the observed titer of the phage on a given BIM host by that on wild-
149 type strain (each plaque assay having been performed independently three times).

150 All BIMs generated were subjected to PCR profiling to confirm their relatedness to the
151 relevant parent strain from which they were derived by CRISPR locus sequencing (26).
152 Primers applied in this study are listed in Table 2. CRISPR PCR conditions were as follows:
153 95 °C x 10 min, followed by 30 cycles of 95 °C x 15 s, 55 °C x 15 s and 72 °C for either 2
154 min 45 s (CRISPR1), 1 min (STB CRISPR2, 3 and 4), or 1 min 30 s (STA CRISPR2 and 3),
155 with a final extension step of 72 °C for 10 min.

156 PCR-generated products were visualised on a 1 % agarose (Fisher Scientific, USA) gel
157 and purified using a JetQuick PCR purification spin kit (Genomed, Lohne, Germany). Sanger
158 sequencing (of all PCR products and plasmids) was performed by MWG Biotech (Eurofins,
159 Ebersberg, Germany) to verify the integrity of all plasmid constructs and to compare the
160 sequences of the CRISPR loci of the BIMs to those of the corresponding parent strain.
161 CRISPRs were assembled using the Seqman program (DNASTar) and the length of the arrays
162 (as well as spacer number) were determined using the online CRISPR finder program (51).
163 Detailed analyses of CRISPR repeat/spacer loci (including all BIM-acquired spacer
164 sequences generated as part of this study) are provided in Supplementary Tables S1, S2 and
165 S6 - S9.

166

167 ***Sedimentation, microscopic and adsorption assays***

168 Relative sedimentation of *S. thermophilus* parent strains and derived BIMs was observed by
169 growing (in liquid culture) representatives of each strain or BIM under identical conditions
170 (as described above). Taking care not to disturb the liquid broth, visual assessment of the
171 cultures was then performed to observe relevant growth characteristics such as pelleting,
172 clumping and adherence to the walls of the tubes. In all cases, this assay was performed using
173 both glass and plastic tubes in order to account for any possible influence of these materials
174 on observed sedimentation patterns.

175 Morphological assessment and comparison of parent strains and derived BIMs was
176 performed via wet mount. 5 μ l of fresh overnight culture was placed on a glass slide (in
177 duplicate) and a cover slip immediately placed on top of the sample. Each sample was then
178 visualised using 63 X magnification using a confocal laser scanning microscope and a Zeiss
179 LSM 5 Exciter (Carl Zeiss, Jena, Germany; excitation 488 nm). The percentage increase in
180 chain length or cells per chain (CPC) of derived BIMs relative to the parent strains was
181 calculated firstly by determining the average number of individual cells per chain in all
182 samples by counting at least 20 chains per strain. The average increase in length was then
183 expressed as a percentage using the following formula: $(CPC_{\text{mutant}} - CPC_{\text{parent}}) / CPC_{\text{parent}} \times 100$
184 (%). In all cases, the unpaired student t-test was used to determine significant differences
185 between the datasets obtained from the parent and its derived BIMs.

186 Adsorption assays were performed as described previously (52), in an adaptation of
187 the protocol as described in a subsequent publication (53).

188

189 ***Construction of antisense plasmid vectors***

190 In order to distinguish *cas* genes associated with distinct CRISPR-Cas systems, a specific
191 nomenclature was devised which was generally in accordance with a previously described
192 naming system (34). Where identically named *cas* genes were found to be associated with

193 distinct CRISPR systems on a single bacterial genome, a suffix was added to denote this (for
194 example, *csn2-1* refers to the gene encoding the Csn2 protein associated with the CRISPR1-
195 Cas system in that strain, etc.). The PCR primers used to amplify *csn2-1*, *csn2-3*, *cas9-1* and
196 *cas9-3* from either STA or STB are listed in Table 2. All primers were designed to
197 incorporate the entire relevant gene including the Shine-Dalgarno (SD) sequence, with the
198 exception of those targeting *cas9-3*, which were designed to incorporate approximately 3 kb
199 of this gene (of a total gene size of 4167 bp), due to the insert size constraint of plasmid
200 pNZ44 (this plasmid becomes unstable when large fragments are cloned into it due to the fact
201 that it is a rolling circle replication plasmid (54)). To ensure that an antisense product was
202 produced, all *cas* genes were cloned into the pNZ44 plasmid in the reverse orientation
203 relative to the p44 promoter (44). In the case of single-gene constructs, the relevant gene was
204 cloned directly behind the p44 promoter in reverse orientation, whereas in the 2Csni construct
205 (simultaneously targeting the *csn2* genes of both the CRISPR1-Cas and CRISPR3-Cas
206 systems, i.e. *csn2-1* and *csn2-3*), *csn2-1* was cloned directly behind the p44 promoter,
207 followed by *csn2-3*, both again in reverse orientation relative to the transcriptional direction
208 of the p44 promoter. PCR amplifications were described as above using the appropriate
209 extension time for each gene. PCR product purification and sequencing was performed as
210 described above. Restriction of plasmid vectors and inserts was performed using the
211 appropriate restriction enzyme (Roche, Basel, Switzerland) in a total volume of 200 μ l
212 overnight at room temperature (RT). Ligations were performed using T4 DNA ligase (New
213 England Biolabs, Ipswich, MA, U.S.A.) in a total volume of 10 μ l overnight at RT. In
214 preparing the 2Csni construct, 3 μ l of each appropriate insert was used in the ligation
215 reaction. Antisense constructs used in this study are listed in Table 1.

216

217 ***Preparation of competent cells, electrotransformation & transformant screening***

218 Competent cells were prepared as described previously (55), with the following
219 modifications: a series of tubes containing 10 ml LM17 or GM17 (for *L. lactis*) broth and
220 varying (from 0.2 % to 2.4 %) concentrations of threonine (Sigma-Aldrich) were prepared
221 and inoculated (1 %) with a fresh overnight culture. The tubes were incubated at 42 °C
222 overnight and examined for growth. LM17 broth containing 0.5 % sucrose (Sigma-Aldrich;
223 SLM17) supplemented with the highest level of threonine tolerated by the strains was used to
224 prepare competent cells.

225 Prior to transformation, plasmid constructs, prepared using the GeneJet plasmid
226 Miniprep kit (Thermo Scientific, Waltham, MA, U.S.A.), were dialysed using 0.025 µm MF
227 membrane filters (Merck Millipore, Billerica, MA, U.S.A.) for 10 mins against sterile
228 distilled water (sdH₂O). All constructs were generated in *L. lactis* NZ9000 prior to their
229 subsequent transfer to *S. thermophilus* STA or STB. Electrotransformation was performed
230 using freshly prepared competent cells as described above, wherein a mixture of cells (50 µl)
231 and plasmid construct (10 µl) was transferred to a pre-chilled (on ice) 2 mm electroporation
232 cuvette (Cell Projects, Kent, U.K.) and subjected to electroporation at 1.75 kV (*S.*
233 *thermophilus*) or 2.0 kV (*L. lactis*)/200 Ohm/25 µF. Following this, 950 µl recovery broth
234 (LM17 or GM17 with the addition of 20 mM MgCl₂ and 2 mM CaCl₂ (Sigma-Aldrich)) was
235 immediately added and the transformed cells were recovered at 30 °C (*L. lactis*) or 42 °C (*S.*
236 *thermophilus*) for 2.5 hrs, prior to spread plating (100 µl) on LM17 or GM17 + Cm5 agar
237 plates as described above. Presumed transformants were purified on LM17 or GM17 + Cm5
238 agar plates and subjected to CRISPR sequencing, phage sensitivity assays (as described
239 above) and plasmid sequencing, which was performed using primers designed outside the
240 MCS of pNZ44 (pNZ44F and pNZ44R; Table 2).

241

242 ***Plasmid curing***

243 To cure pNZ44 and pNZ44-derivative vectors from *S. thermophilus* transformants, such
244 strains were subjected to at least two overnight passages at 42 °C in LM17 in the absence of
245 Cm. Overnight cultures were then ten-fold serially diluted in ¼ strength Ringers solution
246 (Merck), or a culture streak performed, and individual colonies were assessed for sensitivity
247 to Cm by streaking on LM17+Cm5 agar plates. Colonies incapable of growth on
248 LM17+Cm5, yet capable of growth in the absence of Cm were defined as presumptive cured
249 transformants and subjected to validation by CRISPR PCR and plasmid preparation as
250 described above. In cases where two overnight passages were not sufficient for plasmid
251 curing (as indicated by the ability to grow on LM17+Cm5), passaging was repeated until
252 curing was achieved and cured derivatives validated as outlined above.

253 **Results and Discussion**

254 ***BIMs of S. thermophilus STA***

255 ***Generation of CRISPR and non-CRISPR-mediated BIMs of STA***

256 Considering that the two bacterial strains applied in this study were observed to behave
257 differently when subjected to phage attack, distinct *cas* silencing approaches were adopted.
258 For this reason, STA and STB will be discussed separately. BIMs of *S. thermophilus* STA
259 (Table 1) were generated by phage exposure, and validated by phage sensitivity assays and
260 CRISPR locus sequencing (Table 3 and Supplementary Table S1). BIMs of this strain were
261 readily generated in this manner (occurring at a frequency of approximately 1×10^{-5} , estimated
262 by dividing the cfu/ml of visible BIMs after overnight incubation by the cfu/ml of the
263 overnight culture used in the plaque assay), and two BIMs (designated STA BIM1 and STA
264 BIM2; Table 1), both generated against phage 7951, were selected for further
265 characterisation. The relative efficiencies of plaquing (EOP) of each assessed phage on a
266 given BIM are given in Table 3. While STA BIM1 was found to be resistant only to the
267 phage used in the challenge (7951), STA BIM2 was shown to be resistant to all four,
268 genetically distinct (56), phages available against the parent strain (phages 7951, 7952, 7953
269 and 7954, here referred to as phages 7951-4, Table 1). In addition, while presumed ‘escape
270 mutant’ phages were detected on STA BIM1 using the plaque assay method (as indicated by
271 single, well-formed plaques occurring in the bacterial lawn upon challenge using undiluted
272 phage lysate), this was not the case for STA BIM2, for which no escape mutants could be
273 isolated (Table 3). Determination of the sequences of the CRISPR1, 2 and 3 loci of the two
274 BIMs (CRISPR4 is not detected by PCR and presumed to be absent), and comparison to
275 those of the parent strain from which they had been derived, showed that STA BIM1 had
276 acquired a single spacer in the CRISPR1 locus (Supplementary Tables S1 and S7; with
277 schematic representations of the CRISPR1-Cas and CRISPR3-Cas loci given in Figure 1).

278 The sequence of this added spacer displayed 100 % identity to a region on the genome of
279 phage 7951 (partly located within *ORF35*₇₉₅₁ which encodes a predicted RecT recombinase
280 (56), consistent with protospacer targeting (30). In contrast, the sequences of the CRISPR1, 2
281 and 3 loci of STA BIM2 were identical to the parent strain (Supplementary Table S1). Taken
282 together, these data indicate that the mechanisms by which STA BIM1 and STA BIM2 had
283 become resistant to phage 7951 were distinct.

284 To further characterise the derived BIMs, STA BIM1 and STA BIM2 were subjected
285 to adsorption (Table 4), sedimentation (Fig. 3) and morphological assays (Fig. 4, Table 5) as
286 described in the Materials and Methods. Adsorption efficiency of phage 7951 (which was
287 used in the challenge) on STA BIM1 was not significantly different compared to the parent
288 strain. Similarly, compared to the parent strain, this BIM did not show a significantly
289 different sedimentation profile and cell chain length properties, as measured by counting the
290 number of cells within continuous cell chains in a wet mount preparation of the strain ('cells-
291 per-chain' (CPC)). In contrast to this presumed CRISPR-mediated BIM, strain STA BIM2
292 was shown to exhibit a significant reduction in its ability to adsorb phage 7951, indicating
293 that a phage receptor modification is responsible for the observed phage insensitivity of this
294 BIM. Interestingly, this BIM also showed a distinct sedimentation profile relative to the STA
295 WT (and to STA BIM1), in that cells of STA BIM2 were shown to sediment to the bottom of
296 the tube used for standard overnight growth, as visualised in Figure 3. Indeed, this
297 phenomenon has previously been observed in phage adsorption-deficient derivatives of
298 *Lactococcus lactis* (57) and is indicative of a bacterial cell surface alteration. Lastly, a
299 significant increase in cell chain length was observed in STA BIM2 compared to the parent
300 strain and STA BIM1 (Fig. 4, Table 5), indicative of a cell envelope alteration and consistent
301 with the observed sedimentation phenotype (58, 59). The nature of these mutations have been
302 characterised and will be published elsewhere (McDonnell *et. al.*, unpublished data).

303

304 ***CRISPR-Cas silencing restores the sensitivity of CRISPR-mediated BIMs to phages***

305 An antisense mRNA silencing approach for CRISPR inactivation in *S. thermophilus* was
306 performed to confirm that the observed phage resistance in STA BIM1 was conferred by the
307 CRISPR-Cas system, in contrast to the phage resistance observed for STA BIM2. To this
308 end, STA BIM1 and STA BIM2 were transformed using a *cas9*-interfering (Cas9i) antisense
309 plasmid construct as described in the Materials and Methods. The constitutive p44 promoter
310 combined with the cloning, in reverse orientation, of the native STA *cas9* gene was employed
311 to produce *cas9*-antisense mRNA (partly) complementary to the *cas9*-encompassing mRNA.
312 It was reasoned that if this *cas9*-anti-sense RNA hybridized with its sense counterpart it
313 would interfere with translation of *cas9* mRNA and thus with its associated CRISPR-
314 mediated phage resistance. Table 3 shows the relative EOPs of phages on these ‘CRISPR-
315 silenced’ derivatives. It is clear that when BIM STA BIM1 contains the *cas9*-anti-sense
316 producing plasmid (pNZ44+Cas9-1i), phage sensitivity is restored approximately to wild-
317 type (WT) levels, thus demonstrating that this plasmid is capable of effective CRISPR
318 ‘silencing’ by presumed antisense mRNA production. STA BIM1::pNZ44+Cas9-1i was then
319 subjected to plasmid curing as outlined above. Table 3 shows that, when cured of the
320 silencing plasmid, the respective phage resistance profile of STA BIM1 was restored to that
321 observed prior to the introduction of plasmid pNZ44+Cas9-1i. Interestingly, the introduction
322 of plasmid pNZ44 without insert (as a control), seemed to partially increase the sensitivity of
323 STA BIM1 to all four phages (Table 3), although this increased sensitivity did not approach
324 the level of restored sensitivity of STA BIM1::pNZ44+Cas9-1i to phage 7951 using the *cas*
325 silencing approach (Table 3). The reason for this phenomenon is unknown.

326 To confirm that the insensitivity of *S. thermophilus* STA BIM2 was conferred by a
327 resistance mechanism other than CRISPR1, plasmid pNZ44+Cas9-1i was introduced to this

328 BIM and the resulting strain was then subjected to the phage assays outlined above. It is clear
329 from Table 3 that silencing the CRISPR1-Cas system had no significant effect on the ability
330 of phages 7951-4 to produce plaques on this strain, thus supporting the notion that 7951-4
331 insensitivity in this BIM is not mediated by a CRISPR-Cas system.

332

333 *BIMs of S. thermophilus STB*

334 *STB predominantly produces CRISPR-mediated BIMs*

335 BIMs of *S. thermophilus* strain STB were generated and validated by CRISPR locus
336 sequencing (Supplementary Tables S2, S6, S8 and S9). Phages used in each challenge are
337 listed in Table 1, and the relative EOP values of each phage on each BIM are given in Table
338 6. While BIMs with altered and unaltered CRISPR spacer content were readily derived from
339 strain STA (as described above), all analysed BIMs derived from STB (generally arising at an
340 approximate frequency of 10^{-7}) were shown to contain spacer alterations in either the
341 CRISPR1 or CRISPR3 loci (Supplementary Tables S3 and S9; Fig. 2). This phenomenon is
342 illustrated in Supplementary Tables S3 and S9, which shows that 100 % of analysed BIMs
343 derived from STB WT contained an additional spacer in either locus.

344 The observation that phage exposure invariably results in the addition of CRISPR
345 spacers in STB was exploited to produce a BIM containing iterative additions of spacers
346 acquired upon exposure to previously unencountered phages: STB BIM1 was used to isolate
347 STB BIM2, having been exposed to phages 9854 and 9851, independently and respectively.
348 CRISPR spacer content of each BIM relative to parent strain STB is shown in Supplementary
349 Tables S2 and S8. Successive spacer addition was shown to correspond with phage
350 insensitivity, i.e. additional spacers conferred resistance to the phage against which the BIM
351 had been challenged (Table 6, and as previously shown (40)). It is noteworthy that, while
352 presumed phage CRISPR escape mutants (CEMs) were detected in most cases, phage 9854

353 did not produce plaques at a detectable level on STB BIM2. This may be explained by the
354 fact that the added spacer in the CRISPR1 locus of STB BIM2(added subsequent to challenge
355 with phage 9851) also shows 100 % nucleotide identity to phage 9854 (already targeted by a
356 spacer in CRISPR3). It is plausible that the lack of escape mutants is due to the effect of
357 being targeted by two distinct CRISPR-Cas systems through the addition of these two
358 spacers, a phenomenon which has previously been observed using a similar approach (40).

359

360 ***CRISPR-Cas silencing in CRISPR1 and CRISPR3-mediated STB BIMs***

361 To prove the broader feasibility of the silencing method (as outlined above for strain STA),
362 this approach was adopted in STB derivatives STB BIM1 and STB BIM2, purported to be
363 CRISPR-mediated BIMs. The EOP values given in Table 6 clearly show that in STB BIM1
364 harbouring the Cas9-3i construct (or, in the case of STB BIM2, the Cas9-1i construct),
365 sensitivity to phages used in the initial challenge is largely reverted, where this effect is not
366 seen in STB BIM1 and STB BIM2 that harbour the control pNZ44 plasmid.

367 The specificity of this system is highlighted by restoration of the sensitivity of STB
368 BIM2 to phage 9851 (and not 9854) – indicating that only those phages which had previously
369 been targeted by the now silenced CRISPR-Cas system regain the ability to infect. In the case
370 of STB BIM2::pNZ44+Cas9-1i, however, the restoration of sensitivity to phage 9851 is not
371 complete (the EOP of the phage being approximately 10^{-3} relative to STB WT; Table 6). It is
372 possible that the silencing protocol was not operating at optimal efficiency, potentially due to
373 the plasmid being targeted by the CRISPR1 or (more likely, considering the CRISPR1-Cas9-
374 silencing nature of the insert) the CRISPR3-Cas system also being present in this strain.

375

376 ***CRISPR-Cas silencing reduces the incidence of CRISPR spacer addition during BIM***
377 ***generation***

378 Considering the evident spacer addition in analysed BIMs of STB (mentioned above), we
379 endeavoured to inhibit the generation of CRISPR-mediated BIMs in this strain. For this
380 purpose, two silencing plasmid constructs were employed separately. The first
381 (pNZ44+Csn2-1i), was used to target the *csn2-1* gene, i.e. the CRISPR1-Cas system only.
382 The second construct (pNZ44+2Csni) produced a single antisense transcript targeting *csn2-1*
383 and *csn2-3*, i.e. the action of both the CRISPR1-Cas and CRISPR3-Cas systems
384 simultaneously. The encoded product of *csn2-1* has previously been implicated in spacer
385 acquisition in *S. thermophilus* (30) as well as other species (37), and it was hypothesised that
386 *csn2-3* encodes a protein of similar function in the CRISPR3-Cas system, based on its
387 analogous position in the CRISPR3-Cas locus (Fig. 1, Fig. 2, 26, 60). The *csn2* genes were
388 employed for gene silencing purposes in this case (in contrast with *cas9* genes in previous
389 experiments) due to their smaller size, potentially enhancing plasmid stability and enabling
390 the construction of a tandem construct targeting both systems (for reasons discussed in the
391 Materials and Methods). These constructs were introduced separately into STB WT
392 (Supplementary Table S3), after which BIMs were generated against phage 9854.

393 BIMs derived from STB WT, as well as BIM derivatives from STB containing either
394 pNZ44, pNZ44+Csn2-1i (targeting CRISPR1) or pNZ44+2Csni (targeting CRISPR1-Cas +
395 CRISPR3-Cas simultaneously), were then generated. In general, absolute numbers of BIMs
396 growing in the semi-solid agar were lower when STB containing CRISPR-interfering
397 constructs pNZ44+Csn2-1i or pNZ44+2Csni were used, compared with STB WT or
398 STB::pNZ44, though the frequencies of these BIMs were not determined due to the assay-
399 dependent nature of this phenomenon. Following BIM generation, the leader ends
400 (approximately 500 bp) of CRISPRs 1, 3 and 4 repeat/spacer loci in selected BIMs were
401 subjected to Sanger sequencing. CRISPR2 repeat/spacer loci were excluded from this
402 analysis due to its apparent inactivity in this species (26). A summary of BIMs containing

403 CRISPR repeat/spacer loci which showed alterations is shown in Supplementary Table S3,
404 with detailed analysis in Supplementary table S9. It is clear that, while the majority (70 %) of
405 BIMs generated from the WT strain (and plasmid control) acquired spacers in CRISPR1,
406 those generated from the WT strain containing the Csn2-1 interfering plasmid (pNZ44+Csn2-
407 1i) appeared unable to do so (Fig. 2B). This is consistent with previously published results in
408 which *csn2-1* was inactivated (30). Interestingly, the majority (60 %) of these
409 STB::pNZ44+Csn2-1i derived BIMs showed alterations in the CRISPR3 locus, indicating an
410 increase in the ratio of CRISPR3-mediated BIMs (to CRISPR1-mediated BIMs), in the event
411 of a non-functioning CRISPR1 (Fig 2B). This increase in ratio is reflected in the detection of
412 CRISPR3-mediated BIMs derived from the STB WT::pNZ44+Csn2-1i strain (Supplementary
413 Table S9).

414 A total of 33 % of BIMs generated using STB WT::pNZ44+Csn2-1i (harbouring a
415 CRISPR1-interfering plasmid construct) showed no alterations in either of the two analysed
416 CRISPR loci, demonstrating that the generation of non-CRISPR-mediated BIMs was possible
417 in this strain using this method (Supplementary tables S3 and S9). 50 % of BIMs generated
418 using the WT strain harbouring the pNZ44+2Csn1 construct (targeting both the CRISPR1-Cas
419 and CRISPR3-Cas systems; Fig. 2C) showed no alterations in any CRISPR locus, indicating
420 that this construct may be more effective in reducing the acquisition of CRISPR spacers in
421 response to phages in strain STB. One of the BIMs (designated here as STB BIM3) generated
422 using this silencing construct was subjected to further characterisation by CRISPR
423 sequencing (Supplementary Table S2), phage sensitivity assays (Supplementary Table S4)
424 and adsorption assays (Supplementary Table S5). This BIM, despite lacking additional
425 spacers in any CRISPR locus, exhibited a reduction in sensitivity to phages 9851 and 9854
426 (both *cos*-containing phages), while remaining sensitive to 9853 (a *pac*-containing phage;
427 Supplementary Table S4). This BIM can be said to have a wider ranging phage resistance

428 profile relative to first generation CRISPR-mediated BIMs STA BIM1 and STB BIM1,
429 though the reduction in the EOP of phages 9851 and 9854 is slightly less than that exhibited
430 by the CRISPR-based immunity of STB BIM1 to 9854. STB BIM3 was also shown to adsorb
431 phage 9854 at an approximately 20 % reduced level (compared to the wild type strain;
432 Supplementary Table S5). Despite this decrease in adsorption level, this result was not
433 statistically significant, rendering us unable to define STB BIM3 as an adsorption deficient
434 BIM. Nonetheless, in the absence of CRISPR spacer addition as an explanation for the
435 reduction in phage sensitivity (Supplementary table S2 and S4), STB BIM3 may be defined
436 as a non-CRISPR-mediated BIM, establishing the proof of principle of non-CRISPR BIM
437 generation in *S. thermophilus* using the above described silencing protocol.
438

439 **Conclusions**

440 Several mechanisms of phage resistance have been described in *S. thermophilus*, with the
441 CRISPR-Cas system being by far the most widely studied. The level of detailed research into
442 these systems has confirmed their integral role in the resistance *S. thermophilus* to
443 bacteriophages (26, 29, 30, 39, 43) and has now progressed to the characterisation of so-
444 called ‘anti CRISPR proteins’ expressed by certain phages (61).

445 Mills and colleagues (62) generated a selection of BIMs, wherein CRISPR alteration
446 was observed to be an unpredictable process, with different numbers of spacers being
447 acquired (or deleted; indicating an inherent instability in the system) in the same strain - even
448 when similar phages were used in the challenge. This unpredictability as well as the response
449 of the phages to CRISPR spacer acquisition – namely, the modification of single nucleotides
450 on the phage genomes in order to escape CRISPR targeting (40, 42), suggests that further
451 phage robustness may be achieved by utilising other phage resistance mechanisms present in
452 *S. thermophilus* (in combination with CRISPR). Indeed, this has been shown to be extremely
453 effective in *S. thermophilus* by Dupuis and colleagues using a representative strain
454 (DGCC7710; 43). The purpose of the present study was to develop a useful tool which can be
455 utilised to select non-CRISPR-mediated BIMs, which may not be possible using standard
456 BIM generation methods due to the inherent activity of the system.

457 The *cas9/csn2* silencing method outlined herein was successful in preventing spacer
458 acquisition in CRISPR loci during standard BIM generation experiments. Introducing a
459 CRISPR1-associated *csn2-1* targeting plasmid in STB resulted in 66 % of BIMs characterised
460 having acquired spacers in CRISPR3 (and none in CRISPR1), a clear increase in the
461 detection of CRISPR-3-mediated BIMs, compared to those BIMs produced by the WT strain
462 (of which 90 % showed spacer acquisition in the CRISPR1 repeat/spacer locus). To eliminate
463 or at least reduce CRISPR spacer acquisition in either system, it was therefore necessary to

464 target both *csn2-1* and *csn2-3* using a combinatory, anti-sense-mediated silencing plasmid
465 construct. While a number of CRISPR1 spacer acquisitions were detected using this construct
466 (possibly due to the increased competition for the resultant antisense mRNA transcript),
467 employing this method also resulted in the production of a number of apparently non-
468 CRISPR-mediated BIMs.

469 In this study we present data relating to the generation and characterisation of non-
470 CRISPR-mediated BIMs, specifically, those mediated by an apparent adsorption inhibition
471 mechanism. The nature of phage receptors in *S. thermophilus* have been characterised, and
472 proposed to be carbohydrate in nature (63, 64). BIMs which are deficient in phage adsorption
473 have previously been described, having been selected by immuno-labelling followed by flow
474 cytometry (65), mutant library construction followed by phage challenge (7), or by chemical
475 mutagenesis (13). However, these BIMs were not described as having the phenotypic
476 characteristics of the adsorption-deficient BIMs described above, namely sedimentation and
477 increased cell chain length. The non-CRISPR-mediated BIMs generated in this study
478 displayed some advantages over CRISPR-mediated BIMs, in that they were either resistant to
479 a wider range of phages, or to a higher level (with no phage escape mutants being observed
480 during standard plaque assays using STA BIM2). It has furthermore been shown previously
481 that *S. thermophilus* BIMs mediated by both CRISPR and a restriction/modification (R/M)
482 system are resistant to high levels of phages (43), and it was speculated that CRISPR-Cas
483 systems combined with other antiphage mechanisms also exhibit highly protective effects
484 (also suggested by Viscardi *et al.* (65).

485 The method of BIM generation described above offers a number of advantages over
486 traditional methods of derivation. Firstly, no specialised equipment or chemical mutagenesis
487 is required for its implementation. While the method relies on RNA silencing through
488 plasmid transformation in order to decrease the activity of the CRISPR system(s), the plasmid

489 may be removed prior to industrial implementation. Additionally, the use of antibiotic
490 selection is not required once BIM generation has been carried out. This method may also be
491 used to bias the addition of CRISPR spacers towards either the CRISPR1 or CRISPR3
492 repeat/spacer loci. This may reduce the risk of spacer deletion or replacement in second
493 generation CRISPR BIMs, a process which is thought to function in eliminating older (and
494 therefore less useful) spacers, limiting the size of the CRISPR loci (26, 62). In the case of a
495 hypothetical population of BIMs in which the mechanism of resistance is unknown, this
496 method may also be used to rapidly determine if phage insensitivity is conferred by the
497 CRISPR-Cas system alone or by a combination of mechanisms. Lastly, this method may be
498 employed to generate a diverse collection of *S. thermophilus* BIMs which have become
499 resistant to phage by distinct mechanisms of action, for use in industrial strain blends or
500 rotational schemes.

501

502

503 **Figure legends**

504

505 **Figure 1. Schematic representation of the CRISPR1-Cas and CRISPR3-Cas systems of**
506 ***S. thermophilus* STA.**

507

508 **Figure 2. CRISPR spacer addition patterns in STB WT (A), STB::pNZ44+Csn2-1i (B)**
509 **and STB::pNZ44+2Csn1 (C).**

510

511 **Figure 3.** Observed sedimentation of *S. thermophilus* STA parent strain and derived BIMs.
512 (A) STA WT, (B) STA BIM1 (CRISPR BIM), (C) STA BIM2 (non-CRISPR BIM), where (i)
513 indicates a partial clearing of the supernatant broth and (ii) indicates a visible increase in
514 pellet size.

515

516 **Figure 4.** Representative images of cell chains visualised using a confocal laser scanning
517 microscopy. (A) STA WT, (B) STA BIM1 (CRISPR BIM), (C) STA BIM2 (non-CRISPR
518 BIM).

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523

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537

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542

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- 737

738 Table 1. Bacterial strains and bacteriophages applied during this study.
739

Strain / Phage / Plasmid	Description	Source
<u><i>S. thermophilus</i> strains</u>		
STA	Industrial starter parent strain	DSM, Delft, The Netherlands
STB	Industrial starter parent strain	“
STA BIM1	CRISPR-BIM of STA generated against phage 7951	UCC, Cork, Ireland
STA BIM2STA BIM2	Non-CRISPR BIM of STA generated against phage 7951	“
STB BIM1	CRISPR-BIM of STB generated against phage 9854	“
STB BIM2	CRISPR-BIM derivative of STB generated against phage 9851	“
STB BIM3	Non-CRISPR BIM of STB generated against phage 9854	“
<u><i>L. lactis</i> strains</u>		
NZ9000	Transformation host	(49)
<u>Phages</u>		
7951	Lytic <i>pac</i> -containing phage infecting STA	DSM, Delft, The Netherlands
7952	“	“
7953	“	“
7954	“	“
9851	Lytic <i>cos</i> -containing phage infecting STB	“
9853	Lytic <i>pac</i> -containing phage infecting STB	“
9854	Lytic <i>cos</i> -containing phage infecting STB	“
<u>Plasmid constructs</u>		
pNZ44	Transformation vector	
pNZ44+Cas9-1i	STA CRISPR1-Cas9 silencing vector	This study
pNZ44+Cas9-1i	STB CRISPR1-Cas9 silencing vector	“
pNZ44+Cas9-3i	STB CRISPR3-Cas9 silencing vector	“
pNZ44+Csn2-1i	STB CRISPR1-Csn2 silencing vector	“
pNZ44+2Csn1	STB CRISPR1-Csn2 and CRISPR3-Csn2 silencing vector	“

740 Table 2. PCR primers applied during this study. Incorporated restriction sites, where applicable, are underlined. Internal ‘gap closing’ primers
741 are indicated by a ‘g’, and have been applied to all derivatives of the indicated parent *S. thermophilus* strain.
742

Primer name	Sequence (5' - 3')	Target	Size of product (approx; bp)	Source
pNZ44F	CTAATGTCACTAACCTGCCCG	pNZ44 MCS	Template-dependent	This study
pNZ44R	GCTTTATCAACTGCTGCT	“	“	“
STACas9iF	AGCAGCTCTAGAGTCGTTAGAGGGAGGATTAC	STA Cas9	3366	“
STACas9iR	AGCAGCCTGCAGTTAAAAATCTAGCTTAGGC	“	“	“
STBCas9-1iF	AGCAGCTCTAGAGTTGCGAATTTTCAGATAC	STB CRISPR1-Cas9	3366	“
STBCas9-1iR	AGCAGCCTGCAGGTAAGTGTGTAAGGCGCC	“	“	“
STBCas9-3iF	AGCAGCCTGCAGAAGGAGAAATGTATGACTAAG	STB CRISPR3-Cas9	4167	“
STBCas9-3iR	AGCAGCCATGGCTGGCTCTAGTTTAGGGTATT	“	“	“
STBCsn2-1iF	AGCAGCCTGCAGCAGTGATAATAAGTTGGTGGT	STB CRISPR1-Csn2	1053	“
STBCsn2-1iR	AGCAGCCATGGCTGTCTTGTCAATCCTTAC	”	“	“
STBCsn2-3iF	AGCAGCTCTAGAGCCAATTCAGAGGAAAGG	STB CRISPR3-Csn2	660	“
STBCsn2-3iR	AGCAGCCTGCAGCAAGATGTGACTGTACC	”	“	“
CRISPR1F	TGCTGAGACAACCTAGTCTCTC	CRISPR1 repeat/spacer loci	Template-dependent	(26)
CRISPR1R	TAAACAGAGCCTCCCTATCC	“	“	“
CRISPR2F	TTAGCCCTACCATAGTGCTG	CRISPR2 repeat/spacer loci	“	“
CRISPR2R	TAGTCTAACACTTTCTGGAAGC	“	“	“
CRISPR3F	CTGAGATTAATAGTGGGATTACG	CRISPR3 repeat/spacer loci	“	“
CRISPR3R	GCTGGATATTCTGTATAACATGTC	“	“	“
CRISPR4F	GATTCAAGTTCCTCATAGAGC	CRISPR4 repeat/spacer locus	“	This study
CRISPR4R	GACCTCAACCAATCGATTG	“	“	“
AC1g1 (internal)	CCTGTCATCTCTGGGAGT	STA CRISPR1	N/A	“
AC1g2 (internal)	CGGTGTTCTATATCGAGGTC	“	“	“
AC1g3 (internal)	GTGAATGGGAAACTGACGGAA	“	“	“
AC3g1 (internal)	CAATCCGTAGCCACACCT	STA CRISPR3	“	“
BC1g1 (internal)	CACTTGGCAGGCTTATTACTC	STB CRISPR1	“	“
BC1g2 (internal)	CATCCGGTAACTGCTCAAGTG	“	“	“

743

744

745

746 Table 3. Relative efficiencies of plaquing (EOPs) of four phages on STA, its derived BIMs
747 and their CRISPR-Cas silenced derivatives. Values preceded by '≤' indicate the limit of
748 detection of phage titer.

749

	7951	7952	7953
STA WT	1	1	1
STA BIM1	8.33 (± 0.12) x 10 ⁻⁸	0.55 ± 0.16	0.75 ± 0.28
STA BIM1::pNZ44	4.87 (± 0.61) x 10 ⁻⁶	4.95 ± 1.10	6.02 ± 0.68
STA BIM1::pNZ44+Cas9-Ii	1.03 ± 0.07	1.08 ± 0.27	0.93 ± 0.07
STA BIM1::pNZ44+Cas9-Ii (cured)	2.21 (± 3.00) x 10 ⁻⁶	4.08 ± 3.50	4.04 ± 2.76
STA BIM2	≤ 3 x 10 ⁻⁷	≤ 1 x 10 ⁻⁸	≤ 4 x 10 ⁻⁸
STA BIM2::pNZ44+Cas9-Ii	≤ 3 x 10 ⁻⁷	≤ 4 x 10 ⁻⁷	1.85 (± 2.62) x 10 ⁻⁷

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755 Table 4. Adsorption analysis of *S. thermophilus* parent strains and derived BIMs.

756

Strain/BIM	Adsorption of phage 7951 (%)	p-value
STA WT	79.9 ± 13.6	
STA BIM1	83.2 ± 1.8	0.76
STA BIM2	10.2 ± 8.2	0.0034

757

758

759

760 Table 5. Relative cells per chain (CPC) of *S. thermophilus* parent strains and derived BIMs.

761

Strain	CPC (cells)	% CPC average versus parent	p-value
STA (parent)	10.84 ± 8.64 (n=28)	N/A	N/A
STA BIM1	7.4 ± 8.30 (n=32)	68.3	0.12
STA BIM2	25.9 ± 13.0 (n=51)	238.9	3.9 x 10 ⁻⁵

762

763

764 Table 6. Efficiencies of plaquing (EOPs) of phages 9851, 9853 and 9854 on STB, its derived
765 BIMs and their CRISPR-Cas silenced derivatives. Values preceded by '≤' indicate the limit
766 of detection of phage titer.

767

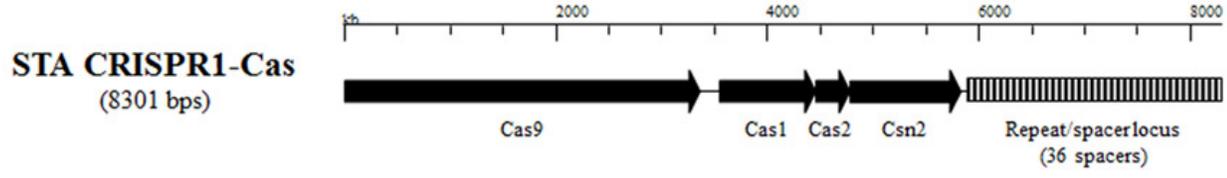
768

	9851	9853	9854
STB WT	1	1	1
STB BIM1	0.22 ± 0.09	0.26 ± 0.06	2.04 (± 0.79) x 10 ⁻⁶
STB BIM1::pNZ44	0.27 ± 0.06	0.30 ± 0.09	4.01 (± 0.79) x 10 ⁻⁶
STB BIM1::pNZ44+Cas9-3i	2.35 ± 0.33	1.15 ± 0.24	0.22 ± 0.08
STB BIM2	9.80 (± 5.93) x 10 ⁻⁷	0.13 ± 0.05	≤ 1 x 10 ⁻⁹
STB BIM2::pNZ44	2.59 (± 1.04) x 10 ⁻⁶	0.23 ± 0.08	≤ 1 x 10 ⁻⁹
STB BIM2::pNZ44+985Cas9-1i	0.002 ± 0.0002	0.56 ± 0.17	≤ 1 x 10 ⁻⁹

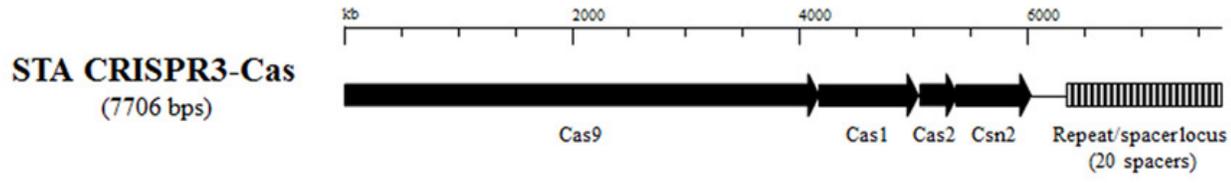
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