Roles of Putative Sodium-Hydrogen Antiporter (SHA) Genes in *S. coelicolor* A3(2) Culture with pH Variation

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Culture pH change has some important roles in signal transduction and secondary metabolism. We have already reported that acidic pH shock enhanced actinorhodin production in *Streptomyces coelicolor*. Among many potential governing factors on pH variation, the putative Na+/H+ antiporter (*sha*) genes in *S. coelicolor* have been investigated in this study to elucidate the association of the *sha* on pH variation and secondary metabolism. Through the transcriptional analysis and overexpression experiments on 8 *sha* genes, we observed that most of the *sha* expressions were promoted by pH shock, and in the opposite way the pH changes and actinorhodin production were enhanced by the overexpression of each *sha*. We also confirmed that *sha8* especially has a main role in maintaining cell viability and pH homeostasis through Na+ extrusion, in salt effect experiment under the alkaline medium condition by deleting *sha8*. Moreover, this gene was observed to have a function of pH recovery after pH variation such as the pH shock, being able to cause the sporulation. However, actinorhodin production was not induced by the only pH recovery. The *sha8* gene could confer on the host cell the ability to recover pH to the neutral level after pH variation like a pH drop. Sporulation was closely associated with this pH recovery caused by the action of *sha8*, whereas actinorhodin production was not due to such pH variation patterns alone.

**Keywords:** Sodium-hydrogen antiporter, culture pH variation, secondary metabolism, *S. coelicolor*

A major proportion of pharmaceutically important secondary metabolites, including antibiotics and anticancer agents, are produced by actinomycetes. Many laboratories are focusing their attention on signal transduction and genetic regulation related to secondary metabolism in actinomycetes. In previous studies on secondary metabolites production by *Streptomyces* strains including *S. coelicolor*, we found that more secondary metabolites were synthesized after a spontaneous or artificial pH drop, with better recovery than when pH changes were suppressed by using a buffer [12–14, 21]. This indicated that culture pH change had some important roles in signal transduction and secondary metabolism. Among a number of potential governing factors on pH regulation, we focused on the Na+/H+ antiporter (sodium-hydrogen antiporter: *sha*) genes for *S. coelicolor* since they are putatively associated with proton transport. In general, *sha* genes are known to play important roles in pH homeostasis, and cell volume regulation in opposition to abrupt pH change [3, 6, 8, 17]. In addition, all living cells actively extrude sodium ions for detoxification, because a high intracellular concentration of sodium ion inhibits many metabolic activities. The major Na+-extruding mechanism in most bacterial cells is known to be the Na+/H+ antiporter (SHA), which exchanges H+ for Na+ [19]. There has been no report on Na+/H+ antiporters in *Streptomyces* strains. However, *S. coelicolor* has in total 8 putative *sha* genes on its chromosome, according to the NCBI database. Each putative *sha* gene has a Na+/H+ or K+/H+ antiporter conserved domain. Sequence alignment results have shown that those genes have homology to many other annotated sodium hydrogen antiporters. To determine whether the putative *sha* genes of *S. coelicolor* are associated with culture pH regulation, their expression levels were analyzed and compared in 3 different culture modes: with suppressed pH changes (pH-controlled; PC), spontaneous pH changes (pH-non-controlled; PNC), and acidic pH shock (pH-shocked; PS). Recombinants were constructed by transforming *S. coelicolor* and *S. lividans* with a high-copy plasmid containing each of the 8 *sha* genes for the overexpression of each of them and investigation of their roles in pH
regulation and secondary metabolism. A deletion mutant and its complement were also constructed for sha8 to confirm its roles (the sha8 gene was selected because it produced the strongest effects).

**MATERIALS AND METHODS**

**Strains and Plasmids**

*S. coelicolor* A3(2) M145 (ATCC BAA471) and *S. lividans* TK24 provided by the John Innes Institute, United Kingdom, were used as transformation host strains. In particular, *S. coelicolor* A3(2) was also used as the model strain for investigation of pH shock effects. *E. coli* DH5α and ET12567 were used for routine subcloning and plasmid amplification [20]. The plasmids of pGEM-T (Promega) and pWHM3, a *Streptomyces*-*E. coli* shuttle vector, were used as cloning and expression vectors, respectively. The latter has a high-copy number with no promoter. For the deletion of the sha8 gene from the chromosome of *S. coelicolor*, pKC1139 and pFDNeo were used together, and for the complementation of this gene, pKUM20 with a low-copy number was used [10, 23]. Characteristics of these strains and plasmids are summarized in Table 1.

**Media and Culture Conditions**

Solid and liquid R2YE media, and Supplemented Minimal Medium Solid (SMMS) [10] were used for cultivation of the *Streptomyces* strains and their transformants. Cultures were grown in a 500 ml baffled flask containing 100 ml of liquid medium at 28°C and at 200 rpm in a rotary shaker. The seed culture was carried out for 2 days under the same conditions, 5 ml of this being used for inoculation. In the cultures a rotary shaker. The seed culture was carried out for 2 days under the same conditions, 5 ml of this being used for inoculation. In the cultures

**PCR Amplification**

Polymerase chain reaction (PCR) amplification was performed using the *Taq* polymerase (TaKaRa, Japan) in a thermocycler (GeneAmp PCR System 2700; Applied Biosystem, CA, USA). After the initial denaturation step (5 min at 96°C), 30 cycles of amplification with 3 steps (30 s at 96°C, 1 min at 60°C, and 1 min at 72°C) were followed by the final extension period of 10 min at 72°C. The pGEthe deletion mutant-easy vector was used for cloning. DNA sequences of the products were routinely verified by TaKaRa Korea Corporation (Seoul, Korea).

**Transformation**

Ampicillin (50 µg/ml), kanamycin (50 µg/ml), and apramycin (25 µg/ml) were used as selection markers for *E. coli* transformation. To avoid restriction of methylated DNA, the dam/dcm *E. coli* strain ET12567 [18] was used for the preparation of plasmids, which were then used to transform *S. coelicolor* and *S. lividans* protoplasts [10]. The desired transformant was selected by overlaying the plate with an aqueous suspension containing thioestrepton (50 µg/ml), apramycin (50 µg/ml), and neomycin (60 µg/ml).

**Construction of sha-Overexpressed Recombinant**

Eight putative sodium-hydrogen antiporter genes on the chromosome of *S. coelicolor* were designated as sha1-sha8 following their SCO numbers in the NCBI database. The coding region including the promoter for each sha gene was PCR-amplified from *S. coelicolor* A3(2) genomic DNA. The primers were supplied by DyneBio Science (Korea). Primers used are F: 5’-CGGCTGGGCGGAGCACCAGG

### Table 1. Strains and plasmids used.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. coelicolor</em> A3(2)</td>
<td>SCP-1, SCP-2, prototroph</td>
<td>Kieser et al. [10]</td>
</tr>
<tr>
<td><em>S. lividans</em> TK24</td>
<td>Str-6</td>
<td>Kieser et al. [10]</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>supE44ΔlacU169(Ø80lacZAM15)</td>
<td>Hanahan et al. [7]</td>
</tr>
<tr>
<td><em>E. coli</em> ET12567</td>
<td>hsdR17 recA1 endA1 gyr96 thi-1 relA1</td>
<td>MacNeil et al. [18]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM</td>
<td><em>E. coli</em> vector, pUC19 backbone, T-cloning flank, amp'</td>
<td>Promega</td>
</tr>
<tr>
<td>pWHM3</td>
<td>High-copy, ts', amp', <em>E. coli</em>–<em>Streptomyces</em> shuttle vector</td>
<td>Vara et al. [22]</td>
</tr>
<tr>
<td>pFDNeo</td>
<td><em>E. coli</em>–<em>Streptomyces</em> shuttle vector neomycin gene, aac(3)I VlacZototiT6K, pSG5, pBR322</td>
<td>Denis et al. [5]</td>
</tr>
<tr>
<td>pKC1139</td>
<td><em>E. coli</em>–<em>Streptomyces</em> shuttle vector low-copy-number</td>
<td>Bierman et al. [1]</td>
</tr>
<tr>
<td>pKUM20</td>
<td><em>E. coli</em>–<em>Streptomyces</em> shuttle vector low-copy-number</td>
<td>Yamazaki et al. [23]</td>
</tr>
</tbody>
</table>

...
CGT-3′ and R: 5′-GGGCCCAAGAGGTACACCGCCGAC-3′ for 
sha8(SC00015); F: 5′-GGCCCAGCAATGCGGCAAGGTGAC-3′ and 
R: 5′-CTGGCCATGTCGCCGACCACCA-3′ for sha2(SC00285); 
F: 5′-CGCCGAGACCTGGTACGACCACGCGG-3′ and R: 5′-CAGTCT 
CTGGCCGAGACCTGGGAGAAGC-3′ for sha8(SC02071); F: 5′-GAAG 
CTGGCTCTGGCAGAACG-3′ and R: 5′-TGAAGGGAGGACAC 
CCCTCGTATGC-3′ for sha8(SC03185); F: 5′-GCGGAGAGGGAG 
GACCTGTGTCGCC-3′ and R: 5′-CCCCTGTTCCTCTGC 
CAG-3′ for sha8(SC03564); F: 5′-CCAGGGAGATATACCCGCTCG 
GIAAGAAGG-3′ and R: 5′-CTACGGCTTGGGCTGATCGCAGATG-3′ for 
sha6(SC03603); F: 5′-GACGGTATGCCCGCCGAGGTACCCGGC-3′ and 
R: 5′-CGCCGAGCCGCGGGCCGGAGACCGG-3′ for sha8(SC05246); 
and F: 5′-GGCCGCGCGCCGCTGGCAGTAGTC-3′ and R: 5′-GAAGC 
GTCGCCGAGCRCACACCCGCGC-3′ for sha8(SC07832).
The PCR-amplified products purified by a DNA Purification 
Kit (TakaRa Korea) were cloned in the pGEMt-easy vector with T4 
ligase (Promega). The resulting recombinant plasmids were 
cloned by using a Miniprep Kit (TakaRa Korea). The recombinant 
plasmids were digested with EcoRI purchased from TaKaRa Korea, 
and the inserts were ligated into the same site on pWHM3. Each of 
the 8 resulting recombinant plasmids, designated pWHM3-sha1 
– pWHM3-sha8, were transformed into S. coelicolor A3(2) and S. 
lividans TK24 for overexpression.

Construction of sha8 (SC07832) Deletion Mutant of S. coelicolor 
A3(2)
For the construction of a plasmid to be used for gene deletion, the 
1 kb fragments at the upstream and downstream regions of the 
sha8 locus were PCR-amplified by using S. coelicolor A3(2) genomic 
DNA as the template. Primer sets used were 5′-AAGCTTAAGGAG 
TGCGTATCTGGTACGAGGC-3′ (the underline indicates a 
HindIII site), 5′-GCAATAGAGCCGACGATGATCCG-3′ (the underline indicates 
a SphI site) for the amplification of the upstream region, 5′- 
CGTCGGGAGCCGCGCCGCTGGTACGAGGC-3′ (the underline indicates 
a KpnI site), and 5′-GAATAGCTGATCGGCCGCGGGCTGGCAAGAAGGAC- 
3′ (the underline indicates an EcoRI site) for the downstream region.
The PCR-amplified products were designated as U and D depending 
on their origins; that is, upstream and downstream of gene 
sha8. They were purified by using a DNA Purification 
Kit (TakaRa Korea) and cloned in the pGEMt-easy vector with T4 
ligase (Promega). The cloned plasmid purified by using a Miniprep Kit (TakaRa Korea) 
was sequenced for verification. The PCR primer set was designed to 
collect HindIII–SphI sites (upstream) and KpnI–EcoRI sites (downstream) 
to facilitate cloning into pFDNeo. The pFDNeo-U site 
was generated by ligating pFDNeo, U, and D was 
digested again by 
HindIII and EcoRI, and then the resulting DNA fragment 
of UNeoD was ligated into pKC1139 to construct pKC1139-UNeoD. 
The plasmid of pKC1139-UNeoD was propagated once in the 
methanol-deficient E. coli strain ET12567 for the preparation of 
non-methylated DNA. It was introduced in S. coelicolor A3(2) by 
protoplast transformation. The plasmid of pKC1139 bears a 
temperature-sensitive Streptomycyes replication origin that cannot 
replicate above 34°C. The transformant containing pKC1139-UNeoD 
was harvested on an R2YE plate containing apramycin and neomycin. 
After being grown for 36 h at 28°C in R2YE liquid medium 
containing neomycin, the transformant cells were transferred to a 
new R2YE liquid medium and cultivated for 48 h at 40°C.
The transformant culture medium was diluted with distilled water 
by a factor 10^3–10^4 before being spread onto a plate of R2YE agar 
medium containing neomycin. The colonies grown were replicated 
on 2 different R2YE plates containing neomycin and apramycin, 
respectively. The true sha8-deletion mutant was expected to be 
resistant to neomycin (Neo+) while having a sensitivity to apramycin 
(Apr+). Correct sha8 gene deletion was checked by both PCR and 
Southern hybridization. 

Confirmation of deletion by PCR. Two primer sets were used. 
One of them, with a designed based on sha8 flanking sequences at both 
ends, consisted of 5′-AGATCTCTTCCCTCAFGT-3′ (forward primer), 5′-TGGTACGACCA-3′ (reverse primer). 
The other set had 5′-GCGGATCATGCGCTTCGCTC-3′ (forward primer), 
5′-GCGTGATCACCGCCGACACGC-3′ (reverse primer). This 
primer set was designed to generate a DNA fragment of 600 bp 
from a DNA sequence in the middle of the sha8 gene.

Confirmation of deletion by Southern hybridization. S. coelicolor 
A3(2)’s chromosomal DNA was digested with restriction enzymes 
of KpnI and BamHI, electrophoresed in a 0.8% agarose gel 
overnight, and blotted to Schleicher and Schuell Nytran membranes. 
The neomycin gene and a 540 bp DNA fragment located upstream 
of sha8 were used as the probe.
Labeling, hybridization, and detection were carried out using a 
Genius 1 Non-radioactive DNA Labelling Kit (Roche Diagnostics 
GmbH).

Investigation of Salt Effects on sha8-Deletion Mutant 
SMMs and Supplemented Minimal Medium Liquid (SMML) 
containing Tris buffer were used to investigate salt effects on the 
growth and actinorhodin production by sha8-deletion mutant 
(sha8) mutant. Spore stocks of wild strain, control strain with only the vector of 
pKC1139, and sha8 mutant (hereafter, the deletion mutant) were 
incubated. Two different initial pHs of 7.3 and 9.0 were tested. In 
each, NaCl of 0–400 mM was added to SMM, which had already 
0.5 mM of Na+ in the form of NaHPO4. Cultivation was carried out at 
28°C for 7 days.

Complementation of the sha8 Gene 
To confirm the role of the sha8 gene, its complementation was 
performed with a low-copy plasmid of pKUM20. A primer set of 
5′-GAAATTCCGGCGCGTTCCCTTCGCGT-3′ (the underline indicates 
a EcoRI site), 5′-AAGCTTGATGCGCCGGACACAC-3′ (the underline indicates a 
HindIII site) was used to PCR-amplify the 
coding region of sha8 from the genomic DNA of S. coelicolor 
A3(2). The PCR-amplified products purified by a DNA Purification 
Kit (TakaRa Korea) were cloned in the pGEMt-easy vector with T4 
ligase (Promega). The cloned plasmid purified by using a Miniprep Kit (TakaRa Korea) 
was sequenced for verification. The recombinant plasmid was digested with EcoRI and 
HindIII, and then the insert was ligated into pKUM20, a SCP2-derived 
Streptomycyes- E. coli shuttle vector with a thiostrepton resistance (Tsf) gene. The resulting plasmid, 
pKUM20-sha8, was introduced into the sha8-deletion mutant for complementation. The transformant with Tsf 
was isolated and used to examine the complementation effects in 
normal and pH-shocked cultures.

Analysis 
Cell and actinorhodin concentrations. Cell concentration was 
measured in dry cell weight (DCW). Cells collected off the 
cellophane film were washed with phosphate buffer. The washed 
cells were dried at 80°C for 24 h before being weighed at room
temperature. The intracellular and extracellular amounts of actinorhodin produced were separately measured following the procedures previously reported [2]. For the analysis of intracellular actinorhodin, 20 mg of dried cells was extracted with 5 ml of chloroform in a test tube for 30 min at room temperature. Then, 5 ml of 1 N NaOH was added. The mixture was vortexed and spun in a microcentrifuge for 15 s. The resulting aqueous phase contained actinorhodin, having a blue color at an alkaline pH of 12. The optical density of the aqueous phase was determined at 615 nm. For the analysis of actinorhodin secreted into agar, the agar was heat-melted before optical density measurement (pH 12 and 615 nm).

**RNA isolation and RT–PCR analyses.** For RNA sample preparation, cells scraped from the cellophane film on SMMS at the different times during development were immediately treated with RNAProtection Bacteria Reagent (Qiagen) for 5 min to stabilize the in vivo transcript profile. After centrifugation, the mycelia pellet was resuspended in 5 ml of RLt buffer (Qiagen), sonicated for 30 s (3 times with an amplitude of 30%), and centrifuged at 13,000 × g for 15 min at 4°C to remove cell debris. The supernatant was extracted in phenol/ chloroform. The aqueous phase was added to 3 ml of ethanol and loaded onto an RNeasy Midi column (Qiagen). The total RNA was quantified using a NanoDrop ND-1000 (Nanodrop, USA). RNA integrity was assessed using a Bioanalyzer (Agilent Technologies).

Transcript detection analysis was carried out by using a Superscript One-Step RT–PCR kit (Invitrogen) with 0.25 µg of total RNA as template. For reverse transcription reaction, the reaction mixes were incubated at 45°C for 30 min, and the reactions were stopped at 95°C for 5 min. The PCR program was as follows: initial denaturation step (5 min at 96°C), annealing step (30 s at 96°C), and elongation step (1 min at 60°C) for 30 cycles. The reaction was completed by incubating for 10 min at 72°C. The samples were separated on 1% agarose gels in TBE buffer and stained with ethidium bromide.

**RESULTS**

**Effect of Culture Mode on sha Genes Expression Pattern**

To investigate the effects of the cultivation mode (PC, PNC, or PS) or pH change pattern on the expression of sha genes, transcription was analyzed by RT–PCR. The results of RT–PCR analysis for the 3 different modes are shown in Fig. 1, along with the pH profiles that have been reported elsewhere [14]. The expressions of 7 sha genes, except sha1, were upregulated by pH shock. The bands of the samples from PS appeared as early as 3 days, 1 day after the pH shock, whereas in PNC and PC, bands appeared after 4 and 5 days, respectively. In PNC, the expressions were mostly found at 4 days, which means 1 day after the induction time of expression in PS.

**Overexpression of sha Genes**

Effects of overexpression of each sha gene on cell growth and secondary metabolite production were observed with the naked eye in solid cultures of the transformants. Only the results for S. lividans are shown in Fig. 2A, since the transformants of S. coelicolor were not significantly different. The control strain (with pWHM3) showed the typical morphology and brown color of S. lividans, whereas 6 transformants with sha2, 3, 4, 5, 7, and 8 genes were a dark blue color, implying a significant amount of actinorhodin production. In particular, the transformants with sha4 and sha8 produced more actinorhodin than the others. The wild type of S. lividans produces no measurable actinorhodin.

To investigate the overexpression effects quantitatively, liquid cultures were prepared by using R2YE medium with no pH buffer. Table 2 shows the cell and actinorhodin concentration profiles for S. coelicolor and S. lividans transformants. Overall, actinorhodin production by the transformants was higher than in the control strains. S. coelicolor transformants with sha4, sha5, sha7, and sha8, had relatively high actinorhodin productivity of over 0.3 OD_{595nm}/(g-DCW/l), and S. lividans transformants with sha4 and sha8 were over 0.1 OD_{595nm}/(g-DCW/l). Growth of most transformants, except both strains with sha3, was comparable to that of the control culture. Fig. 2B shows the pH profiles in the culture of S. lividans transformants. The pH initially dropped from 7.3 to ~6 in the control culture, and then recovered to 8 with time. Whereas the transformant with sha7 showed a similar pH pattern to the control, those with sha1, 2, 3, 4, 5, and 8 changed more dramatically, although the strain with sha6 showed no notable variations in pH.

**Construction and Confirmation of sha8-Deletion Mutant**

As mentioned above, 2 transformants with sha4 and 8 overexpression showed the most notable differences in pH profiles and actinorhodin production from the wild type. For this reason, we had tried to construct 2 deletion
mutants deprived of sha4 and 8 as a way of confirming the role of SHA. However, when the sha4-deletion was constructed, we did not obtain any colony. Since the cell did not grow, the sha4-deletion mutant could not be screened.

To delete the chromosomal sha8 gene by double-crossover, a recombinant plasmid of pKC1139-UNeoD was constructed. The plasmid of pKC1139 carrying Apra' was a conjugative plasmid, which can be expressed in both E. coli and Streptomyces. This plasmid consisted of the E. coli origin of ori T and one Streptomyces ori from pSG5. The ori fragment from pSG5 contains a temperature-sensitive replicon, so that pKC1139 could not replicate

Table 2. Cell and actinorhodin concentrations in liquid culture of sha-overexpressed transformants.

<table>
<thead>
<tr>
<th></th>
<th>S. lividans  TK24</th>
<th>S. coelicolor  A3(2)</th>
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<tbody>
<tr>
<td></td>
<td>Cell conc. (g-DCW/l)</td>
<td>ACT conc. ((g-DCW/l)^2)</td>
</tr>
<tr>
<td>Only pWHM3</td>
<td>1.25</td>
<td>0.0416</td>
</tr>
<tr>
<td>pWHM3+sha1</td>
<td>0.83</td>
<td>0.0646</td>
</tr>
<tr>
<td>pWHM3+sha2</td>
<td>1.75</td>
<td>0.0423</td>
</tr>
<tr>
<td>pWHM3+sha3</td>
<td>2.28</td>
<td>0.0753</td>
</tr>
<tr>
<td>pWHM3+sha4</td>
<td>1.98</td>
<td>0.1253</td>
</tr>
<tr>
<td>pWHM3+sha5</td>
<td>1.75</td>
<td>0.0441</td>
</tr>
<tr>
<td>pWHM3+sha6</td>
<td>1.44</td>
<td>0.0209</td>
</tr>
<tr>
<td>pWHM3+sha7</td>
<td>1.48</td>
<td>0.0932</td>
</tr>
<tr>
<td>pWHM3+sha8</td>
<td>1.68</td>
<td>0.1220</td>
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</table>
above 34°C. As a result of double-crossover, 3 colonies with selective markers of Neo and Apra were obtained by replacing sha8 with UNeo’d. For confirmation, PCR amplifications using 2 different types of primer sets were performed. One primer set was designed based on sha8 flanking sequences at both ends. The other primer set was designed to generate a DNA fragment of 600 bp existing in the sha8 gene, as mentioned above. As expected with using the first primer set, a single band of 1.2 kb corresponding to the length of gene sha8 was recognized on the genomic DNA of the wild strain by PCR, whereas a single band of 1.0 kb corresponded to Neo’ in the sha8-deletion mutant. Using the second primer set, a single band was detected around 600 bp in the wild-type strain, but this was not present in the mutant. Further confirmation of sha8 deletion came from Southern hybridization analysis. As expected, in the first case, the DNA fragment of 2.39 kb was detected with a Neo probe only in the mutant sample, and in the second, fragments of 2.39 kb and 12.62 kb were detected in mutant and wild-type samples, respectively. These results clearly indicated that sha8 had been successfully replaced with Neo’.

Cultivation of sha8-Deletion Mutant
Salt and pH effects on cell growth. To confirm the main function of sodium-hydrogen antiporter, a simple growth study for the sha8-deletion mutant was undertaken by varying the amount of NaCl in the medium to 0.5, 100.5, 200.5, and 400.5 mM. Experiments were carried out at neutrality (pH 7.3) and in alkali solution (pH 9.0). Fig. 3 shows the photographs of plates and cell concentration data for solid cultures of the wild type and the deletion mutant. Growth of both strains overall increased with the salt concentration and showed lower values at pH 9.0 than at neutral pH. In particular, the growth of the deletion mutant was inhibited strongly in 0.5 mM salt at neutral pH (Fig. 3A), whereas no growth occurred irrespective of the salt concentration in alkaline pH (Fig. 3B). Liquid culture experiments also gave similar results (data not shown), although the effects of NaCl concentration and pH were less prominent than in the solid cultures.

Implementation of pH shock. In order to examine the role of sha8, an acidic pH shock was given to solid cultures of the sha8-deletion mutant. The pH and actinorhodin concentration profiles in the culture with no pH control (PNC) and the pH-shocked culture (PS) are shown in Fig. 4A. The pH in PNC of the wild type decreased to 5.0, and then recovered steadily to the neutral level. In the culture of the deletion mutant, however, the pH spontaneously decreased to around 5.5 with no recovery thereafter to the neutral level. The results of PS are shown in Fig. 4B. In the wild-type culture, the pH increased dramatically soon after the pH shock to reach the neutral level in 3 days and eventually a slightly alkaline level of 8. In the deletion mutant, a delay

![Fig. 3. NaCl and pH effects on cell growth of the wild type (WT) S. coelicolor A3(2) and the sha8-deletion mutant (MT). Photographs of solid cultures at pH 7.3 (A) and pH 9.0 (B); and cell concentrations at pH 7.3 (C) and pH 9.0 (D). The values are the means and standard deviations of five independent experiments.](image-url)
in pH recovery was observed. Actinorhodin production by the deletion mutant was higher than that of the wild type in both PNC and PS (Fig. 5). Both strains showed a 5-fold increase in productivity in PS than in PNC.

**DISCUSSION**

**SHA and pH Variation**

First, 3 different modes of cultures were carried out with the wild type of *S. coelicolor* to determine whether SHA is affected by the pattern of pH change during the culture. We observed that an external significant pH variation condition like pH shock culture mode induced the expressions of 7 *sha* genes, except *sha1*, earlier than in the other 2 culture modes of PNC and PC (Fig. 1). In the opposite way, to investigate the role of each *sha* gene on pH regulation and actinorhodin production, the overexpression experiments were carried out in both *S. coelicolor* and *S. lividans*. Because *S. lividans* is phylogenetically very close to *S. coelicolor* and does not produce actinorhodin in normal condition, despite having an actinorhodin biosynthetic gene cluster, this strain was considered to be the best as a host strain for testing the gene dosage effect. Moreover, we previously reported the effects of pH shock were quite similar in both strains. Thus, we introduced the *sha* genes originated from *S. coelicolor* into *S. lividans* as well as into itself, because by this we expected to give similar or more dramatic effects. The pH in the cultures of most *S. lividans* and *S. coelicolor* transformants decreased to acidic level, even without an acidic pH shock, and recovered to the neutral range (Fig. 2B). Such pH variation was much more significant than in its control. The overexpressed *sha* gene seemed to create a situation similar to that with pH shock overall. The *shal* showed, however, a significant effect on culture pH variation in the overexpression strain although
it was not expressed by the pH shock. This result implies that \(sha1\) gene might have the role not to recover the medium pH after pH shock implementation but to decrease mainly the medium pH. The two sets of experimental results discussed above clearly indicated that most \(sha\) genes were associated with pH regulation. Furthermore, the overexpression of a certain \(sha\) gene had similar effects to pH shock on the actinorhodin production. In particular, actinorhodin production was markedly enhanced by overexpression of \(sha4\) and \(sha8\), in both host strains (Table 2). Along with the evidence above that these \(sha\) genes were associated with pH regulation and actinorhodin biosynthesis, an in-depth study was performed with a deletion mutant to elucidate the role of the \(sha\) gene more clearly.

**Salt and pH Effects on \(Sha8\)-Deletion Mutant**

Since overexpressed \(sha4\) and \(sha8\) genes showed the most prominent effects on pH regulation and secondary metabolite biosynthesis, we had tried to construct 2 different deletion mutants deprived of \(sha4\) or \(sha8\) genes. Unfortunately, only the \(sha8\)-deletion mutant could be constructed. It was speculated that the deletion of the \(sha4\) gene was too detrimental for the cell to survive.

The result that the growth of deletion mutant depends on the salt concentration, being inhibited by alkaline pH of medium, indicates that the function of \(sha8\) is \(Na^+\)-dependent. Similar results have been reported elsewhere [17]. According to this report, cell growth increased with \(Na^+\) concentration when the salt concentration was low. However, when the \(Na^+\) concentration was high enough, growth was notably repressed in alkaline conditions. To date, the \(sha\) genes have been annotated only by the sequence alignment in the genome project of NCBI for \(S. coelicolor\). No data about this function have been reported yet. Thus, we performed the salt effect experiments at pH 9.0 for the first time in \(S. coelicolor\) to confirm efficiently the function of the \(sha\) gene as a salt efflux pump. Such alkaline condition was established in order to generate a reversed pH difference between inside and outside of the cells, which should increase the efflux of \(Na^+\) by SHA. All living cells are known to actively extrude \(Na^+\) by SHA and maintain an inwardly directed gradient of sodium ions [9, 15–17]. When the medium pH is very high, passive transport of protons from inside of the cell to the outside becomes significant. To compensate for this proton loss and maintain pH homeostasis, cells are expected to actively pump out sodium ions and thus take in more protons, which consume more energy. For this reason, growth was expected to retard at the alkaline pH compared with that at the neutral level. As expected, growth at pH 9.0 was generally lower than at pH 7.3 (Fig. 3). The deletion mutant, in particular, never grew at alkali pH, irrespective of the salt concentration. The higher the medium pH, the more important became the role of SHA, as discussed above. However, the \(sha8\)-deletion mutant, with a weakened SHA function, was considered to be unable to fulfil such an enhanced requirement and thus perished. Therefore, our results imply that pH homeostasis by \(sha8\) is very important for cell viability, especially in alkaline conditions, although the other \(sha\) genes survived.

**Effects of pH Changes on Cultures of the \(sha8\)-Deletion Mutant and the Complement**

Finally, to elucidate the action of \(sha8\) on pH changes and its involvement in secondary metabolism, including spore formation and actinorhodin biosynthesis, solid cultures of the \(sha8\)-deletion mutant were grown in 2 different culture modes of PNC and PS. As seen in Fig. 4, the deletion mutant showed the pH recovery retardation in both cultures compared with the wild-type strain. This result strongly supports that \(sha8\) plays an important role in pH recovery or regulation in addition to the evidence that we mentioned in the previous discussion. Hence, we thought that an ability of pH recovery in the deletion mutant would be restored by complementing \(sha8\). However, unexpectedly, when an acidic pH shock was applied, the pH was very rapidly recovered with no time delay to reach the initial level in one day just after the shock, and then decreased and increased again to eventually reach pH 8. From these results, we were also convinced that its role could be maximized when there was an external pH disturbance, as in the PS mode culture. In addition, although not mentioned under Results, the \(sha8\)-deletion mutant did not grow in a pH-controlled culture (PC) for unknown reasons, whereas the complementation strain showed normal growth. This is another piece of evidence that \(sha8\) is closely associated with pH variation in the culture.

Based on our previous study [14], we had expected that actinorhodin production by the deletion mutant and the complementation strain would be lower than in the wild type, since the former 2 showed a less significant pH variation in PNC than the latter, but the results conflicted with this expectation. Moreover, in PS, actinorhodin production by the wild type or the deletion mutant was enhanced by pH shock. Actinorhodin production by the complementation strain with the pH shock, however, was unexpectedly low, despite showing very significant pH variations. These results suggest that the extent of actinorhodin biosynthesis could not be explained by culture pH variation alone. However, we observed that spore formation was closely associated with the phenomenon of pH recovery since the spores were generated only after the pH had recovered to neutral pH (data not shown). According to a previous study similar to our observation, pH recovery to neutral pH promoted the expression of sigma H as well as its relevant genes known to be involved in sporulation [4].
In conclusion, expression of sha genes in *S. coelicolor* A3(2) was observed to be strongly induced by an acidic pH shock. We have also observed from our previous study that acidic pH shock enhanced the actinorhodin production remarkably. To determine the relationships among pH variation such as a pH shock, expression of *sha* genes, and actinorhodin production, a series of experiments of overexpression of *sha* genes, and deletion and complementation of *sha8*, one of the 8 *sha* genes, were performed. It has been clearly shown that *sha8* plays a major role in regulating the pH after pH variation. However, no clear conclusions emerged on whether *sha8* is associated through pH regulation, directly or indirectly, with actinorhodin production. Only sporulation was closely associated with pH recovery by the action of *sha8*.

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**References**