Characterization of clinically isolated thymidine-dependent small-colony variants of *Escherichia coli* producing extended-spectrum β-lactamase

Tatsuya Negishi,¹,² Takehisa Matsumoto,³,* Kazuki Horiuchi,¹ Eriko Kasuga,¹ Tatsuya Natori,¹ Mina Matsuoka,¹ Naoko Ogiwara,¹ Mitsutoshi Sugano,¹ Takeshi Uehara,¹ Noriyuki Nagano² and Takayuki Honda¹

**Abstract**

**Purpose.** Thymidine-dependent small-colony variants (TD-SCVs) are difficult to detect or test for antimicrobial susceptibility. We investigated the characteristics of clonal TD-SCVs of *Escherichia coli*, both with and without *blaCTX-M-3*, isolated from a patient.

**Methodology.** Mutation in the thyA gene was analysed by sequencing, and morphological abnormalities in the colonies and cells of the isolates were examined. Additionally, conjugational transfer experiments were performed to prove the horizontal transferability of plasmids harbouring resistance genes.

**Results.** The TD-SCVs contained a single nucleotide substitution in the thyA gene, c.62G>A, corresponding to p.Arg21His. Morphologically, their colonies were more translucent and flattened than those of the wild-type strain. In addition, cells of the TD-SCVs were swollen and elongated, sometimes with abnormal and incomplete divisions; a large amount of cell debris was also observed. Changing c.62G>A back to the wild-type sequence reversed these abnormalities. Conjugational transfer experiments showed that the TD-SCV of *E. coli* with *blaCTX-M-3* failed to transfer *blaCTX-M-3* to *E. coli* CSH2. However, the TD-SCV of *E. coli* without *blaCTX-M-3* experimentally received the plasmid encoding *blaSHV-18* from *Klebsiella pneumoniae* ATCC 700603 and transferred it to *E. coli* CSH2.

**Conclusion.** Mutation in the thyA gene causes morphological abnormalities in the colonies and cells of *E. coli*, as well as inducing thymidine auxotrophy. In addition, TD-SCVs horizontally transmit plasmids encoding resistance genes. It is important to detect TD-SCVs based on their characteristics because they serve as reservoirs of transferable antibiotic resistance plasmids.

**INTRODUCTION**

Small-colony variants (SCVs) is a collective term for mutant strains that grow slowly and show atypical colony morphology [1, 2]. SCVs are associated with persistent and recurrent infections [3, 4]. Thymidine-dependent small-colony variants (TD-SCVs) require thymidine for their growth, and TD-SCVs have been reported in many species such as *Staphylococcus aureus* [5, 6], methicillin-resistant *S. aureus* [7], *Enterococcus faecalis* [8, 9], *Escherichia coli* [9–11] and *Proteus mirabilis* [9, 11]. TD-SCVs are likely to be isolated from patients who have received trimethoprim-sulfamethoxazole treatment for a long period [5–11] because TD-SCVs are resistant to this antimicrobial agent. Trimethoprim-sulfamethoxazole interferes with dihydropteroate synthase and dihydrofolate reductase, which are involved in the synthesis and conversion of tetrahydrofolic acid, a cofactor of thymidylate synthase [12]. In many cases, TD-SCVs have mutations in the thymidylate synthase-encoding gene; the wild-type enzyme catalyses the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate. As a result of these mutations, TD-SCVs cannot synthesize thymidine, which is required for DNA synthesis and bacterial replication; these strains

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**Keywords:** small-colony variants; thymidine-dependent; ESBL; *Escherichia coli*.

**Abbreviations:** ESBL, extended-spectrum β-lactamase; MH, Mueller Hinton; NCV, normal-colony variant; SCV, small-colony variant; SEM, scanning electron microscopy; TD-SCV, thymidine-dependent small-colony variant.

Two supplementary tables and four supplementary figures are available with the online version of this article.
therefore take up thymidine from the environment [1, 13]. TD-SCVs cannot grow on Mueller Hinton (MH) media because of their low thymidine content [5]. Accordingly, drug-resistant TD-SCVs are particularly problematic because of the associated challenges in detection during susceptibility testing using MH media.

Characteristics of TD-SCVs have been mainly studied in \textit{S. aureus}. In \textit{S. aureus}, the mutation in \textit{thyA}, the structural gene for thymidylate synthase, is the main cause for thymidine auxotrophy [6, 12, 14, 15]; some TD-SCVs possess the ability to revert to normal-colony variants (NCVs) that show normal phenotypes and can grow on MH media during passage culture [6, 16]. TD-SCVs of \textit{S. aureus} also show abnormalities in their colony and cellular morphologies compared with wild-type strains [17]. However, the characteristics of TD-SCVs of other species, including \textit{E. coli}, have not been sufficiently elucidated to date.

Extended-spectrum \( \beta \)-lactamases (ESBLs) are a group of \( \beta \)-lactamases (including TEM-, SHV-, OXA- and CTX-M types) that are produced mainly by \textit{Enterobacteriaceae}. ESBLs are capable of hydrolysing a wide range of \( \beta \)-lactam antibiotics, including third-generation cephalosporins and monobactams, thus making the development of therapies against their infections challenging [18, 19]. In most cases, the genes responsible for ESBL production are located on plasmids, and are therefore transferable from strain to strain and between bacterial species. The emergence of an SCV of ESBL-producing \textit{Klebsiella pneumoniae} has been recently reported [20].

In this study, we examined the genetic and morphological changes of TD-SCVs of \textit{E. coli} isolated from faeces and urine of a patient, and investigated the conjugational trans-ferability of the ESBL genes in these isolates.

**METHODS**

**Bacterial strains**

Three SCVs (SCV-4474, SCV-4478 and SCV-4539) were isolated from a patient with congenital atresia of the biliary system at Shinshu University Hospital, Japan. SCV-4474 and SCV-4478 were isolated from the faeces and urine, respectively, on the same day, and SCV-4539 was isolated from faeces 27 days later. Biochemical identification with the Api 20E system (Sysmex bioMérieux) proved unsuccessful; therefore, 16S rRNA gene sequence analysis was used for the identification of all strains as \textit{E. coli} [21]. The possibility of the isolates representing \textit{Shigella} species was ruled out due to evidence of lactose degradation.

NCVs of the three SCVs, namely NCV-4474, NCV-4478 and NCV-4539, were successfully established by passage culture using MH media. All three SCVs and the three NCVs were shown to be clonal by PFGE using XbaI for total DNA digestion [22] (Fig. S1, available in the online version of this article).

\textit{K. pneumoniae} ATCC 700603 containing \textit{bla}_{SHV-18} [23] and \textit{E. coli} CSH2 \((\textit{metB} \text{ F} \text{ Rif}^+ \text{ Na}^+)\) were used in the conjugational transfer experiments as the donor strain and recipient strain, respectively (Table 1).

**Auxotrophy testing**

A McFarland No. 0.5 suspension of the SCVs was prepared and spread on MH agar (BD Japan). Autoclaved filter paper containing 10 µg thymidine, hemin and menadione was then placed on the agar, followed by incubation overnight at 35 °C.

**PCR and DNA sequencing**

The ORF of the \textit{thyA} gene (795 bp) of the SCVs and NCVs was amplified and sequenced using primers Ec \textit{thyA}-F (5'-TTCCATCCCGATGATTGTC-3') and Ec \textit{thyA}-R (5'-GGTCTCGAAAGATTTAAC-3'), which were designed based on the \textit{thyA} sequences of \textit{E. coli} ATCC 25922 (accession number CP009072) and \textit{E. coli} ATCC 8739 (accession number CP000946).

The ESBL genes in the SCVs were screened by PCR with specific primers for the \textit{bla}_{CTX-M-1} group, \textit{bla}_{CTX-M-2} group, \textit{bla}_{CTX-M-9} group, \textit{bla}_{TEM} and \textit{bla}_{SHV} [24, 25]. The complete ORF sequence of the ESBL gene was amplified and sequenced [26]. The primers used for PCR and sequencing are listed in Table S1.

For sequence determination, the amplicons were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics), and both strands were directly sequenced using a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype and/or phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Escherichia coli} CSH2</td>
<td>Faeces</td>
<td>Thymidine-dependent</td>
</tr>
<tr>
<td>SCV-4474</td>
<td>Faeces</td>
<td>Revertant of SCV-4474</td>
</tr>
<tr>
<td>NCV-4474</td>
<td>Urine</td>
<td>Thymidine-dependent</td>
</tr>
<tr>
<td>SCV-4478</td>
<td>Faeces</td>
<td>Revertant of SCV-4478</td>
</tr>
<tr>
<td>NCV-4478</td>
<td>Faeces</td>
<td>Thymidine-dependent</td>
</tr>
<tr>
<td>SCV-4539</td>
<td>Faeces</td>
<td>Revertant of SCV-4539</td>
</tr>
<tr>
<td>NCV-4539</td>
<td>Faeces</td>
<td>Donor strain possessing \textit{bla}_{SHV-18} used in conjuga- tional transfer experiments</td>
</tr>
<tr>
<td>\textit{Klebsiella pneumoniae} ATCC 700603</td>
<td>Faeces</td>
<td>Recipient strain used in conjuga- tional transfer experiments (\textit{metB} \text{ F} \text{ Rif}^+ \text{ Na}^+)</td>
</tr>
</tbody>
</table>
BigDye Terminator cycle sequencing ready reaction kit and a 3500 Genetic Analyzer (Thermo Fisher Scientific). The nucleotide and deduced amino acid sequences were analysed using the Basic Local Alignment Search Tool program (http://www.ncbi.nlm.nih.gov/blast). The Clustal W program (http://www.ebi.ac.uk/clustalw) was used to align the amino acid sequences.

**Colony morphology**

The SCVs and NCVs were cultured overnight on trypticase soy agar with 5% sheep blood (BD Japan) at 35°C, and the morphological characteristics were compared with those of *E. coli* ATCC 25922.

**Gram staining and scanning electron microscopy**

Bacterial cells of the SCVs and NCVs were analysed by Gram staining and scanning electron microscopy (SEM) (JSM-7600F; JEOL), and compared with those of *E. coli* ATCC 25922.

**Antimicrobial susceptibility testing**

The MICs of the SCVs were determined using an Etest device (Sysmex bioMérieux) on MH agar with 5% sheep blood (BD Japan) [7, 14]. The MICs of the NCVs were also determined using an Etest, on both MH agar and MH agar with 5% sheep blood. The following antimicrobials were tested: ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, cephalothin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, gentamicin, amikacin, tetracycline and ciprofloxacin. ESBL phenotype was confirmed using the cefotaxime-clavulanate Etest ESBL strip. *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 were used as the quality control strains following the instructions of the manufacturers of the Etest.

**Growth curve analysis of thymine-dependent growth**

Growth curve analysis was performed in accordance with a previous report [27]. The overnight culture of SCVs was suspended in MH broth with different concentrations of thymidine (0, 0.1, 1, 10 and 100 µg ml⁻¹), followed by incubation with shaking (220 r.p.m.) at 35°C. *E. coli* ATCC 25922 and NCVs were tested under the same conditions in MH broth without thymidine for comparison. The OD₆₀₀ values were measured every hour for 12 h. Measurements under each condition were performed three times.

**Conjugational transfer experiments**

Conjugational transfer experiments were performed based on the conjugational mating technique with a minor change: thymidine was used [28]. Briefly, the recipient and donor strains were grown to a mid-logarithmic phase in 1 ml of LB broth supplemented with thymidine (10 µg ml⁻¹). The concentration of thymidine was determined based on the result of growth curve analysis and a previous report [27]. Five hundred microlitres of each bacterial suspension was mixed and centrifuged to yield a bacterial pellet; this pellet was incubated for 6 h at 35°C. Transconjugants were selected with each kind of LB agar containing antimicrobial agents as follows.

**(A) SCV-4539 to *E. coli* CSH2**

SCV-4539, which harbours *blaCTX-M-3*, was used as the donor strain, and *E. coli* CSH2 as the recipient strain. After co-culture, transconjugants were selected on LB agar plates containing ampicillin (100 µg ml⁻¹), rifampicin (100 µg ml⁻¹) and thymidine (10 µg ml⁻¹).

**(B) *K. pneumoniae* ATCC 700603 to SCV-4474**

*K. pneumoniae* ATCC 700603, which harbours *blaSHV-18*, was used as the donor strain, and SCV-4474 as the recipient strain. After co-culture, transconjugants were selected on LB agar plates containing ampicillin (100 µg ml⁻¹), amikacin (4 µg ml⁻¹) [29] and thymidine (10 µg ml⁻¹). The transconjugant was also streaked on trypticase soy agar with 5% sheep blood and MH agar to confirm thymidine auxotrophy. The *blaSHV-18* of the transconjugant was confirmed by PCR with *blaSHV*-specific primers [25].

**(C) SCV-4474 transconjugant carrying *blaSHV-18* to *E. coli* CSH2**

SCV-4474 transconjugant carrying *blaSHV-18*, obtained in experiment (B), was used as the donor strain, and *E. coli* CSH2 as the recipient strain. After co-culture, transconjugants were selected on LB agar plates containing ampicillin (100 µg ml⁻¹), rifampicin (100 µg ml⁻¹) and thymidine (10 µg ml⁻¹). The *blaSHV-18* of the transconjugants was confirmed by PCR with *blaSHV*-specific primers [25].

**RESULTS**

**Auxotrophy and thyA mutation**

Growth of the SCVs around the filter paper containing thymidine was clearly enhanced; however, this effect was not observed around the paper with hemin and menadione (Fig. S2).

In the three SCVs, an identical single nucleotide substitution was found in the *thyA* gene (c.62G>A), which caused a missense mutation (p.Arg21His). However, in all the NCVs, the mutated *thyA* gene sequence changed back to the wild-type *thyA* gene sequence.

**Changes in colony and cell morphologies**

Colonies of the SCVs were more translucent and flattened than those of *E. coli* ATCC 25922, while those of the NCVs were sleek, greyish and elevated, and could not be distinguished from colonies of *E. coli* ATCC 25922 (Fig. 1a).

Gram staining and SEM revealed that the bacterial cells of the SCVs were heterogeneous, swollen and elongated, with abundant cell debris in the background, compared with cells of *E. coli* ATCC 25922. Moreover, many of the particularly swollen cells showed constriction typical of incomplete cell division. The constrictions were not always in the middle and there were sometimes multiple constrictions in a single cell. In contrast, most of the cells of the NCVs were of the
same size as those of *E. coli* ATCC 25922; however, some elongated cells remained even after five passages on MH agar (Fig. 1b, c).

**Antimicrobial susceptibility testing and ESBL confirmation**

SCV-4478 and SCV-4539 showed resistance to cefotaxime, intermediate resistance to aztreonam, and susceptibility to cefoxitin and amoxicillin-clavulinate. Clavulanic acid decreased the MIC of cefotaxime by \( \frac{1}{8} \) two-fold dilutions (Table 2). The MICs of the SCVs did not differ by \( >2 \) two-fold dilutions compared with those of the respective NCVs, both on MH agar and on MH agar with 5% sheep blood (Table S2).

PCR confirmed that SCV-4478 and SCV-4539 possessed the \( \text{bla}_{\text{CTX-M-1}} \) group, but SCV-4474 did not. The sequence of the \( \text{bla}_{\text{CTX-M-1}} \) group completely matched that of \( \text{bla}_{\text{CTX-M-3}} \) (879/879 bp) (accession number Y10278) [30]. PCR amplification for the \( \text{bla}_{\text{CTX-M-2}} \) group, \( \text{bla}_{\text{CTX-M-9}} \) group, \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) could not be performed in SCV-4474, SCV-4478 and SCV-4539.

**Determination of thymidine concentration required for SCV growth**

The SCVs showed a similar growth curve as *E. coli* ATCC 25922 and the NCVs in MH broth supplemented with thymidine at 10 and 100 µg ml\(^{-1}\). Although their log phase was longer than that of *E. coli* ATCC 25922 and NCVs, the final density was almost the same. However, growth of the SCVs showed low optical density with thymidine at 0, 0.1 and 1 µg ml\(^{-1}\) (Fig. 2).

**Conjugational transfer of plasmid encoding the ESBL genes**

Colony growth of the transconjugants on selection agar was not observed in experiment (A) from SCV-4539 to *E. coli* CSH2. In contrast, the transconjugants grew on selection agar in experiment (B), from *K. pneumoniae* ATCC 700603 to SCV-4474, and in experiment (C), from the transconjugant of SCV-4474 carrying \( \text{bla}_{\text{SHV-18}} \) to *E. coli* CSH2. The transconjugants in experiments (B) and (C) were found to be \( \text{bla}_{\text{SHV}} \)-positive in PCR (Fig. S3). The transconjugant in experiment (B) was confirmed to not grow on MH agar.
Table 2. MICs of antimicrobial agents for SCVs

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (µg ml⁻¹) SCV-4474</th>
<th>SCV-4478</th>
<th>SCV-4539</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>6</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Amoxicillin-clavulanate</td>
<td>12</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>8</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>24</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>12</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.125</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Cefotaxime-clavulanate</td>
<td>0.5</td>
<td>0.094</td>
<td>0.064</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>0.5</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0.094</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0.125</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.25</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1.5</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>Amikacin</td>
<td>12</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.004</td>
<td>0.003</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Fig. 2. Thymidine concentration-dependent growth kinetics of SCV-4539, NCV-4539 and E. coli ATCC 25922. Results are expressed as the mean±SD of three experiments. TD, thymidine; OD, optical density.

DISCUSSION

We examined the genetic and morphological changes in TD-SCVs of E. coli isolated from clinical specimens, and investigated the conjugal transferability of the ESBL genes in this study. We found that a mutation in the thyA gene causes thymidine auxotrophy and morphological abnormality in E. coli TD-SCVs. In addition, we revealed that the TD-SCVs of E. coli conjugally transfer drug resistance genes, such as ESBL genes, which indicates that TD-SCVs represent reservoirs of transferable antibiotic resistance plasmids.

All the TD-SCVs in our study harbour the p.Arg21His mutation in thymidylate synthase; this mutation was derived from c.62G>A in the thyA gene. This single amino acid mutation was converted back to arginine, but not to other amino acids, in all the NCVs. According to crystal structure analysis of thymidylate synthase, Arg21, Arg126, Arg127 and Arg166 are well-conserved amino acids, and these four conserved arginine side-chains are relevant to hydrogen bonding interactions with the substrate [31, 32]. Consequently, it is reasonable to interpret that the reduction in the activity of thymidylate synthase was caused by the p.Arg21His mutation, suggesting that, as in S. aureus, the mutation in the thyA gene is responsible for thymidine auxotrophy in E. coli.

We additionally found that the SCV colonies were morphologically abnormal. The colonies of TD-SCVs of S. aureus have been previously reported to be either ‘fried-egg’-like, with translucent edges surrounding a smaller, elevated pigmented centre, or pinpoint in shape [17]. In the present isolates, the colonies of the SCVs were more flattened and translucent than those of wild-type E. coli; the NCV colonies could not be distinguished from those of wild-type E. coli. It was evident that the TD-SCVs of E. coli form morphologically abnormal colonies; however, these isolates could not be identified as SCVs based only on colony morphology. Impaired growth on MH media serves as a more definitive indicator for the identification of TD-SCVs of E. coli.

The SCV cells were abnormally swollen and elongated with impaired cell division and abundant cell debris. These characteristics resemble those of TD-SCVs of S. aureus [17]. The size and shape of the bacterial cells of the NCVs returned almost to normal; however, it was unclear why some of the swollen and elongated cells observed in the SCVs remained in the NCVs even after several passages on MH agar. One possible explanation is that the NCVs retained factors, other than the thyA mutation, that caused morphological abnormalities in bacterial cells.

SCV-4478 and SCV-4539 exhibited ESBL-producing phenotypes and were shown to possess bla<sub>CTX-M-3</sub>. Interestingly, in contrast to SCV-4478 and SCV-4539, SCV-4474 did not possess bla<sub>CTX-M-3</sub>, despite the fact that these SCVs were considered to be originally clonal based on the result of PFGE. One possible explanation for these differences is that some E. coli cells may have acquired the plasmid encoding bla<sub>CTX-M-3</sub> before or after mutating to TD-SCVs. Another equally plausible explanation is that some E. coli cells producing ESBL may have lost the plasmid encoding bla<sub>CTX-M-3</sub> before or after mutating to TD-SCVs. However, we thought the clinically important issue here was not these differences, but the ability of TD-SCVs to spread drug resistance genes such as ESBL.

Our conjugal experiments revealed that TD-SCVs horizontally transmit resistance genes. In these experiments, we added thymidine at 10 µg ml⁻¹ in broth based on the growth curve analysis of thymidine concentration-dependent growth, and in accordance with a previous report [27]. We had hypothesized that the bla<sub>CTX-M-3</sub>-carrying plasmid is transferred from SCV-4539 to E. coli CSH2 by conjulation; however, the present conjugal transfer experiments were unsuccessful. On the other hand, the plasmid-encoded
**Thymidine auxotrophy and morphological abnormalities of TD-SCVs of *E. coli**

Thymidine auxotrophs of *E. coli* are caused by a mutation in the *thyA* gene. Furthermore, it was shown that plasmids carrying drug resistance genes may be transferred via TD-SCVs. This information, in addition to the fact that the TD-SCVs are difficult to detect via susceptibility testing using MH media, highlights the need for the development of methods for the detection of TD-SCVs through routine microbiology testing.

**References**


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**Conflicts of interest**

The authors declare that there are no conflicts of interest.


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