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 Nagano,[2] and Takayuki Honda[1]

[1]Department of Laboratory Medicine, Shinshu University Hospital, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan; [2]Department of Health and Medical Sciences, Shinshu University Graduate School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan; [3]Department of Laboratory Sciences, Gunma University Graduate School of Health Sciences, 3-39-22 Showa-machi, Maebashi, Gunma 371-8514, Japan

*Correspondence: Takehisa Matsumoto

E-mail: tmatumoto@gunma-u.ac.jp

Phone: +81-27-220-8940

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Abbreviations: ESBL, extended-spectrum β-lactamase; MH, Mueller Hinton; MIC, minimum inhibitory concentration; NCV, normal-colony variant; OD, optical density; SCV, small-colony variant; SEM, scanning electron microscopy; TD-SCV, thymidine-dependent small-colony variant.

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 $bla_{\text{CTX-M-3}}$, isolated from a patient.

Methodology.

Mutation in the *thyA* gene was analyzed by sequencing, and morphological abnormalities in the colonies and cells of the isolates were examined. Further, conjugational transfer experiments were performed to prove the horizontal transferability of plasmids harboring 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, the 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 $bla_{\text{CTX-M-3}}$ failed to transfer $bla_{\text{CTX-M-3}}$ to *E. coli* CSH2. However, the TD-SCV of *E. coli* without $bla_{\text{CTX-M-3}}$ experimentally received the plasmid encoding $bla_{\text{SHV-18}}$ 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 *Staphylococcus 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 trimethoprime-sulfamethoxazole treatment for a long period [5-11] because TD-SCVs are resistant to the antimicrobial agent. Trimethoprime-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 catalyzes 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 therefore take up thymidine from the environment [1,13]. TD-SCVs cannot grow on Mueller Hinton (MH) media because of its 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 *S. aureus*. In *S. aureus*, the mutation in *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 *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 *E. coli*, have not been sufficiently elucidated to date.

Extended-spectrum β-lactamases (ESBLs) are a group of β-lactamases (including TEM-, SHV-, OXA-, and CTX-M types) that are produced mainly by Enterobacteriaceae. ESBLs are capable of hydrolyzing a wide range of β-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 therefore transferable from strain to strain and between bacterial species. The emergence of an SCV of ESBL-producing *Klebsiella pneumoniae* has been recently reported [20].

In this study, we examined the genetic and morphological changes of TD-SCVs of *E. coli* isolated from feces and urine of a patient, and investigated the conjugational transferability 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 feces and urine, respectively, on the same day, and SCV-4539 was isolated from feces 27 days later. Biochemical identification with Api 20E (Sysmex bioMérieux Co. Ltd., Tokyo, Japan) proved unsuccessful; therefore, 16S rRNA gene analysis was used for the identification of all strains as *E. coli* [21]. The possibility of the isolates being *Shigella* spp. 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 proved to be clonal by pulsed-field gel electrophoresis using XbaI for total DNA digestion [22] (Fig. S1).

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

Auxotrophy testing

McFarland No. 0.5 suspension of the SCVs was prepared and spread on MH agar (BD Japan Co., Ltd., Tokyo, Japan). Autoclaved filter paper containing 10 μg of 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 *thyA* gene (795 bp) of the SCVs and NCVs was amplified and sequenced using primers Ec thyA-F 5'-TTCCATCCCGATGATTGTC-3' and Ec thyA-R 5'-AAGGYGTCTCGAAGAATTTAAC-3', which were designed based on the *thyA* sequences of *E. coli* ATCC 25922 (Accession Number CP009072) and *E. coli* ATCC 8739 (Accession Number CP000946).

The ESBL genes in the SCVs were screened by PCR with specific primers for the $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-2}}$ group, $bla_{\text{CTX-M-9}}$ group, bla_{TEM}, and 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 Co., Tokyo, Japan), and both strands were directly sequenced using a BigDye Terminator cycle sequencing ready reaction kit and a 3500

Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The nucleotide and deduced amino acid sequences were analyzed using the Basic Local Alignment Search Tool program (http://www.ncbi.nlm.nih.gov/blast). The ClustalW 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 Co., Ltd., Tokyo, 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 analyzed by Gram staining and scanning electron microscopy (SEM) (JSM-7600F, JEOL Ltd, Tokyo, Japan), and compared with those of *E. coli* ATCC 25922.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the SCVs were determined using Etest (Sysmex bioMérieux Co. Ltd., Tokyo, Japan) on MH agar with 5% Sheep Blood

(BD Japan Co., Ltd., Tokyo, Japan) [7,14]. The MICs of the NCVs were also determined using 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 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), followed by incubation with shaking (220 rpm) at 35°C. *E. coli* ATCC 25922 and NCVs were tested under the same conditions in MH broth without thymidine for comparison. The values of optical density at 600 nm (OD_{600}) 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). The concentration of thymidine was determined based on the result of growth curve analysis and a previous report [27]. Five hundred microliters of each bacterial suspension were mixed and centrifuged to yield a bacterial pellet; this pellet was incubated 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 harbors $bla_{\text{CTX-M-3}}$, 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), rifampicin (100 μg/mL), and thymidine (10 μg/mL).

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

K. pneumoniae ATCC 700603, which harbors $bla_{\text{SHV-18}}$, 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), amikacin (4 μg/mL) [29], and

thymidine (10 μg/mL). The transconjugant was also streaked on Trypticase Soy Agar with 5% Sheep Blood and MH agar to confirm thymidine auxotrophy. The bla_{SHV-18} of the transconjugant was confirmed by PCR with bla_{SHV}-specific primers [25].

(C) SCV-4474 transconjugant carrying bla_{SHV-18} to *E. coli* CSH2

SCV-4474 transconjugant carrying bla_{SHV-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), rifampicin (100 μg/mL), and thymidine (10 μg/mL). The bla_{SHV-18} of the transconjugants was confirmed by PCR with bla_{SHV}-specific primers [25].

RESULTS

Auxotrophy and *thyA* mutation

The growth of the SCVs around the filter paper containing thymidine was obviously 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

The colonies of the SCVs were more translucent and flattened than those of *E. coli* ATCC 25922, while those of the NCVs were sleek, grayish, 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 numerous cell debris in the background, compared with *E. coli* ATCC 25922 cells. 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-clavulanate. Clavulanic

acid decreased the MIC of cefotaxime by ≥ 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 MH agar with 5% Sheep Blood (Table S2).

PCR confirmed that SCV-4478 and SCV-4539 possessed the $bla_{\text{CTX-M-1}}$ group, but SCV-4474 did not. The sequence of the $bla_{\text{CTX-M-1}}$ group completely matched that of $bla_{\text{CTX-M-3}}$ (879/879 bp) (Accession number Y10278) [30]. PCR amplification for the $bla_{\text{CTX-M-2}}$ group, $bla_{\text{CTX-M-9}}$ group, bla_{TEM}, and bla_{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 10 and 100 μg/mL of thymidine. Although their log phase was longer than that of *E. coli* ATCC 25922 and NCVs, the final density was almost the same. However, the growth of the SCVs showed low optical density in 0, 0.1, and 1 μg/mL of thymidine (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 $bla_{\text{SHV-18}}$ to *E. coli* CSH2. The transconjugants in experiments (B) and (C) were found to be bla_{SHV}-positive in PCR (Fig. S3). The transconjugant in experiment (B) was confirmed to not grow on MH agar.

DISCUSSION

We examined the genetic and morphological changes in TD-SCVs of *E. coli* isolated from clinical specimens, and investigated the conjugational 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 harbored 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 into Arg, 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 the case of *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 center 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 numerous 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 to almost normal; however, it was unclear as to 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 proved to possess $bla_{\text{CTX-M-3}}$. Interestingly, in contrast to SCV-4478 and SCV-4539, SCV-4474 did not possess $bla_{\text{CTX-M-3}}$, despite the fact that these SCVs were considered to be originally clonal based on the result of pulsed-field gel electrophoresis. One possible explanation for these differences is that some *E. coli* cells may have acquired the plasmid encoding $bla_{\text{CTX-M-3}}$ 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 $bla_{\text{CTX-M-3}}$ 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 conjugational experiments revealed that TD-SCVs horizontally transmit resistant genes. In these experiments, we added 10 μg/mL of thymidine 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*$_{\text{CTX-M-3}}$–carrying plasmid is transferred from SCV-4539 to *E. coli* CSH2 by conjugation; however, the present conjugational transfer experiments were unsuccessful. On the other hand, the plasmid-encoded *bla*$_{\text{SHV-18}}$ was successfully transferred from *K. pneumoniae* ATCC 700603 to SCV-4474, and from this SCV-4474 to *E. coli* CSH2 (Fig. S4). One of the possible reasons for the discrepancy is the difference in the kind of plasmid on which each of the ESBL genes was harbored. It is possible that the plasmid on which *bla*$_{\text{SHV-18}}$ was encoded was transmissible, and that on which *bla*$_{\text{CTX-M-3}}$ was encoded was not [33]. Nevertheless, our results reveal that TD-SCVs transfer plasmid-encoded ESBL genes, and suggest that TD-SCVs are capable of transmitting other drug resistance genes, such as that encoding carbapenemase, harbored on plasmids.

Clinically, drug-resistant TD-SCVs pose significant threats owing to their horizontal spread of drug resistance genes and the challenges associated with susceptibility testing for these strains using MH media. In order to detect TD-SCVs, the elucidation of their characteristics and establishment of methods for susceptibility testing is critical. To date, the characteristics of TD-SCVs have been mainly studied in *S. aureus*. More recently, studies focusing on antimicrobial susceptibility testing in SCVs of *S. aureus*, including

TD-SCVs, have been reported [34]. However, such investigations have not been performed in other species, including Enterobacteriaceae. In this study, we reported the genetic and morphological abnormalities of TD-SCVs of *E. coli*; however, the characteristics exhibited by the present strains may not be characteristic of all TD-SCVs. In the future, further investigations of SCVs in a wide range of species, using additional strains, are required for accurate detection of TD-SCVs.

In conclusion, this study revealed that thymidine auxotrophy and morphological abnormalities in TD-SCVs of *E. coli* are caused by a mutation in the *thyA* gene. Further, 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.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Abbreviations

ESBL, extended-spectrum β-lactamase; MH, Mueller Hinton; MIC, minimum inhibitory concentration; NCV, normal-colony variant; OD, optical density; SCV, small-colony variant; SEM, scanning electron microscopy; TD-SCV, thymidine-dependent small-colony variant.

References

1. **Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P *et al*.** Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* 2006;4(4):295-305.

2. **Melter O, Radojevič B**. Small colony variants of *Staphylococcus aureus*--review. *Folia Microbiol (Praha)* 2010;55(6):548-558.

3. **Sendi P, Proctor RA**. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends Microbiol* 2009;17(2):54-58.

4. **Roggenkamp A, Sing A, Hornef M, Brunner U, Autenrieth IB *et al*.** Chronic prosthetic hip infection caused by a small-colony variant of *Escherichia coli*. *J Clin Microbiol* 1998;36(9):2530-2534.

5. **Gilligan PH, Gage PA, Welch DF, Muszynski MJ, Wait KR**. Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. *J Clin Microbiol* 1987;25(7):1258-1261.

6. **Besier S, Zander J, Siegel E, Saum SH, Hunfeld KP *et al*.** Thymidine-dependent *Staphylococcus aureus* small-colony variants: human pathogens that are relevant not only in cases of cystic fibrosis lung disease. *J Clin Microbiol* 2008;46(11):3829-3832.

7. **Maduka-Ezeh A, Seville MT, Kusne S, Vikram HR, Blair JE *et al*.** Thymidine auxotrophic *Staphylococcus aureus* small-colony variant endocarditis and left ventricular assist device infection. *J Clin Microbiol* 2012;50(3):1102-1105.

8. **Haltiner RC, Migneault PC, Robertson RG**. Incidence of thymidine-dependent enterococci detected on Mueller-Hinton agar with low thymidine content. *Antimicrob Agents Chemother* 1980;18(3):365-368.

9. **Barker J, Healing D, Hutchison JG**. Characteristics of some co-trimoxazole-resistant Enterobacteriaceae from infected patients. *J Clin Pathol* 1972;25(12):1086-1088.

10. **Tanner EI, Bullin CH**. Thymidine-dependent *Escherichia coli* infection and some associated laboratory problems. *J Clin Pathol* 1974;27(7):565-568.

11. **King CH, Shlaes DM, Dul MJ**. Infection caused by thymidine-requiring, trimethoprim-resistant bacteria. *J Clin Microbiol* 1983;18(1):79-83.

12. **Kriegeskorte A, Block D, Drescher M, Windmüller N, Mellmann A *et al*.** Inactivation of *thyA* in *Staphylococcus aureus* attenuates virulence and has a strong impact on metabolism and virulence gene expression. *MBio* 2014;5(4):e01447-01414.

13. **Zander J, Besier S, Saum SH, Dehghani F, Loitsch S *et al*.** Influence of dTMP on the phenotypic appearance and intracellular persistence of *Staphylococcus*

aureus. *Infect Immun* 2008;76(4):1333-1339.

14. **Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA**. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *Int J Med Microbiol* 2007;297(4):217-225.

15. **Chatterjee I, Kriegeskorte A, Fischer A, Deiwick S, Theimann N *et al.*** In vivo mutations of thymidylate synthase (encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. *J Bacteriol* 2008;190(3):834-842.

16. **Besier S, Zander J, Kahl BC, Kraiczy P, Brade V *et al.*** The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* 2008;52(6):2183-2189.

17. **Kahl BC, Belling G, Reichelt R, Herrmann M, Proctor RA *et al.*** Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. *J Clin Microbiol* 2003;41(1):410-413.

18. **Pitout JD, Laupland KB**. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis*

2008;8(3):159-166.

19. **Paterson DL, Bonomo RA**. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* 2005;18(4):657-686.

20. **Ronde-Oustau C, Lustig S, Dupieux C, Ferry T, group LBS**. Implant-associated ESBL-*Klebsiella pneumonia* producing small colony variant bone and joint infection in a healthy 40-year-old man. *BMJ Case Rep* 2017;2017.

21. **Neilan BA, Jacobs D, Del Dot T, Blackall LL, Hawkins PR *et al.*** rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. *Int J Syst Bacteriol* 1997;47(3):693-697.

22. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE *et al.*** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995;33(9):2233-2239.

23. **Rasheed JK, Anderson GJ, Yigit H, Queenan AM, Doménech-Sánchez A *et al.*** Characterization of the extended-spectrum beta-lactamase reference strain, *Klebsiella pneumoniae* K6 (ATCC 700603), which produces the novel enzyme SHV-18. *Antimicrob Agents Chemother* 2000;44(9):2382-2388.

24. **Shibata N, Kurokawa H, Doi Y, Yagi T, Yamane K *et al.*** PCR classification

of CTX-M-type beta-lactamase genes identified in clinically isolated gram-negative bacilli in Japan. *Antimicrob Agents Chemother* 2006;50(2):791-795.

25. **Yagi T, Kurokawa H, Shibata N, Shibayama K, Arakawa Y**. A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. *FEMS Microbiol Lett* 2000;184(1):53-56.

26. **Dutour C, Bonnet R, Marchandin H, Boyer M, Chanal C *et al.*** CTX-M-1, CTX-M-3, and CTX-M-14 beta-lactamases from *Enterobacteriaceae* isolated in France. *Antimicrob Agents Chemother* 2002;46(2):534-537.

27. **Horiuchi K, Matsumoto T, Ota Y, Kasuga E, Negishi T *et al.*** Addition of thymidine to culture media for accurate examination of thymidine-dependent small-colony variants of methicillin-resistant *Staphylococcus aureus*: a pilot study. *J Microbiol Methods* 2015;110:40-44.

28. **Yagi T, Kurokawa H, Senda K, Ichiyama S, Ito H *et al.*** Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like beta-lactamase genes. *Antimicrob Agents Chemother* 1997;41(12):2606-2611.

29. **Zhang J, Keller K, Takemoto JY, Bensaci M, Litke A *et al.*** Synthesis and combinational antibacterial study of 5"-modified neomycin. *J Antibiot (Tokyo)* 2009;62(10):539-544.

30. **Gniadkowski M, Schneider I, Pałucha A, Jungwirth R, Mikiewicz B *et al.*** Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. *Antimicrob Agents Chemother* 1998;42(4):827-832.

31. **Matthews DA, Appelt K, Oatley SJ, Xuong NH**. Crystal structure of *Escherichia coli* thymidylate synthase containing bound 5-fluoro-2'-deoxyuridylate and 10-propargyl-5,8-dideazafolate. *J Mol Biol* 1990;214(4):923-936.

32. **Matthews DA, Villafranca JE, Janson CA, Smith WW, Welsh K *et al.*** Stereochemical mechanism of action for thymidylate synthase based on the X-ray structure of the covalent inhibitory ternary complex with 5-fluoro-2'-deoxyuridylate and 5,10-methylenetetrahydrofolate. *J Mol Biol* 1990;214(4):937-948.

33. **Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EP, de la Cruz F**. Mobility of plasmids. *Microbiol Mol Biol Rev* 2010;74(3):434-452.

34. **Precit MR, Wolter DJ, Griffith A, Emerson J, Burns JL *et al.*** Optimized In Vitro Antibiotic Susceptibility Testing Method for Small-Colony Variant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2016;60(3):1725-1735.

Table 1. Bacterial strains used in this study

Strain	Source	Genotype and/or phenotype
Escherichia coli		
SCV-4474	Feces	Thymidine-dependent
NCV-4474	-	Revertant of SCV-4474
SCV-4478	Urine	Thymidine-dependent
NCV-4478	-	Revertant of SCV-4478
SCV-4539	Feces	Thymidine-dependent
NCV-4539	-	Revertant of SCV-4539
Klebsiella pneumoniae ATCC 700603	-	Donor strain possessing $bla_{\text{SHV-18}}$, used in conjugational transfer experiments
Escherichia coli CSH2	-	Recipient strain used in conjugational transfer experiments (*metB* F^- Rif^r Nal^r)

Table 2. Minimum inhibitory concentrations (MICs) of antimicrobial agents for small-colony variants (SCVs)

	MIC (μg/mL)		
Antimicrobial agent	SCV-4474	SCV-4478	SCV-4539
Ampicillin	6	> 256	> 256
Amoxicillin-clavulanate	12	8	6
Ampicillin-sulbactam	8	48	12
Cephalothin	24	> 256	> 256
Cefoxitin	12	4	3
Cefotaxime	0.125	24	24
Cefotaxime-clavulanate	0.5	0.094	0.064
Ceftazidime	0.5	3	1.5
Cefepime	0.094	6	8
Aztreonam	0.125	6	6
Imipenem	0.25	0.19	0.19
Gentamicin	1.5	128	1
Amikacin	12	4	4
Tetracycline	1.5	1.5	1.5
Ciprofloxacin	0.004	0.003	0.003

Figure and table legends

Fig. 1. Colony and bacterial cell morphologies of the small-colony variants (SCVs) and normal-colony variants (NCVs)

Features of SCV-4539 (I), NCV-4539 (II), and *E. coli* ATCC 25922 (III) on Trypticase Soy Agar with 5% Sheep Blood (a), as analyzed by Gram staining, visualized at 1,000 × magnification (b) and by SEM with 5,000 × magnification (c) are shown.

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

Table S1. Oligonucleotide primers used in PCR and sequencing

Table S2. Minimum inhibitory concentrations (MICs) of antimicrobial agents for normal-colony variants (NCVs)

SB, Sheep Blood; MHA, Muller Hinton agar

Fig. S1. Pulsed-field gel electrophoresis of the small-colony variants (SCVs) and normal-colony variants (NCVs)

Lanes: M, Marker; 1, SCV-4474; 2, SCV-4478; 3, SCV-4539; 4, NCV-4474; 5, NCV-4478; 6, NCV-4539

Fig. S2. Auxotrophy testing

Overnight incubation of SCV-4539 at 35°C on Mueller Hinton (MH) agar with each nutrient

Fig. S3. *bla*$_{SHV}$ PCR of transconjugants

Lanes: M, Size marker; 1, *Klebsiella pneumoniae* ATCC700603; 2, SCV-4474; 3, Transconjugant in experiment (B); 4, *Escherichia coli* CSH2; 5, Transconjugant in experiment (C); 6, No template control

Fig. S4. Conjugational transfer of plasmids encoding ESBL genes

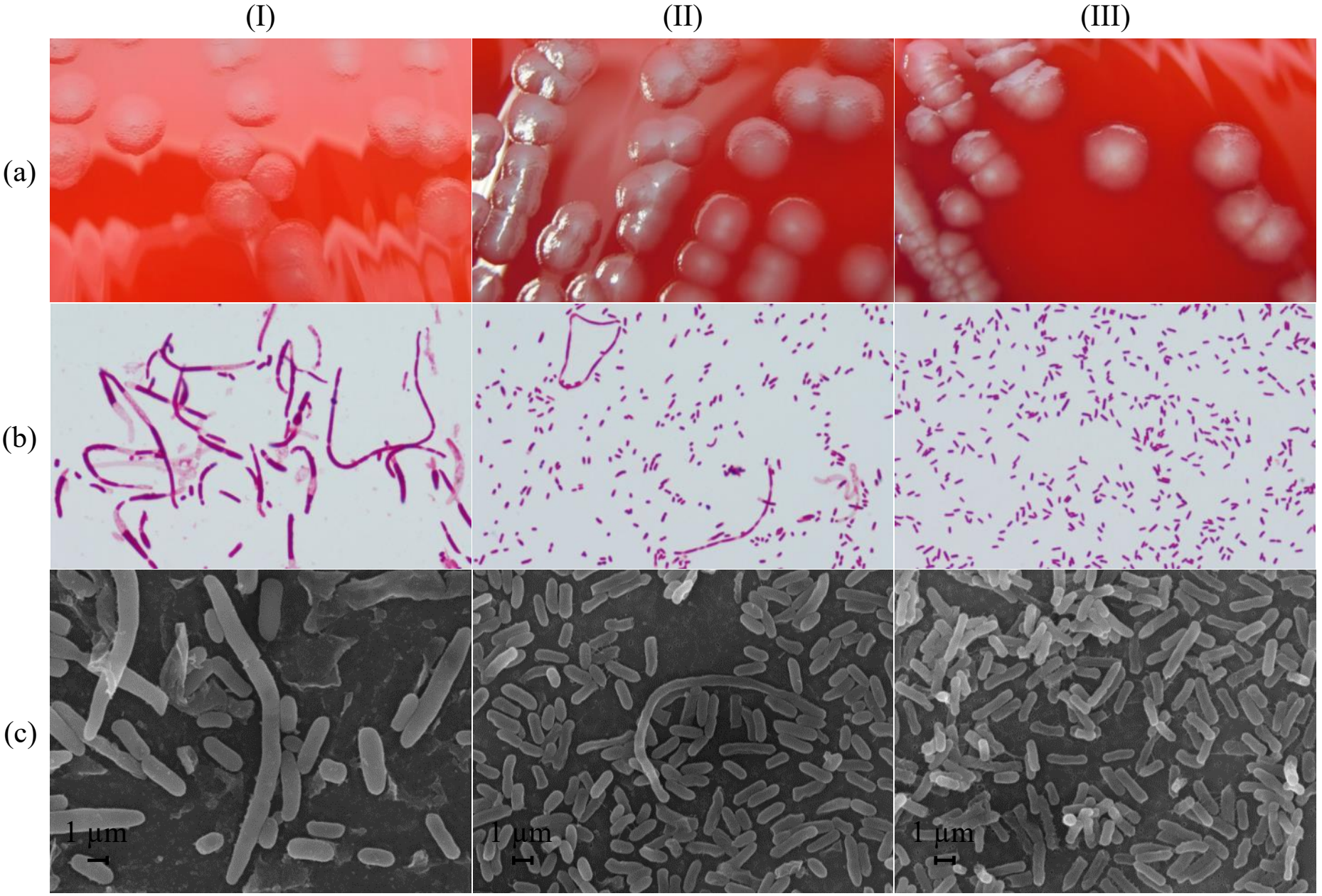

Fig. 1

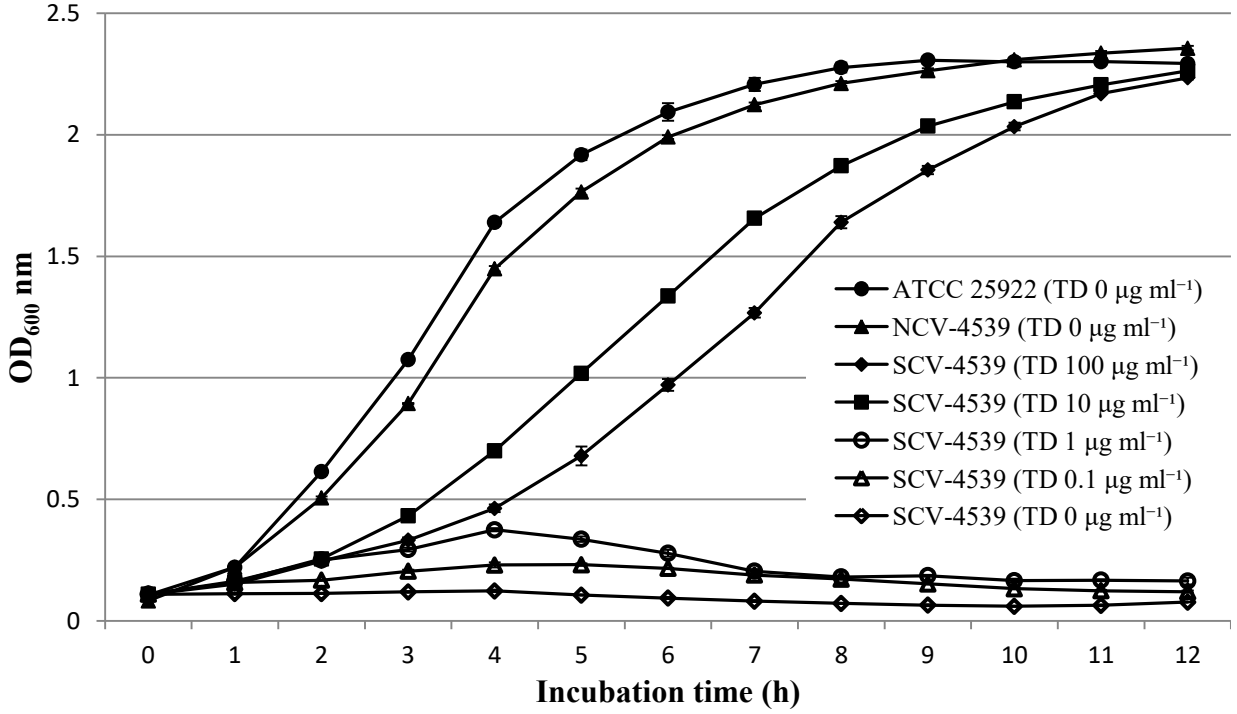
2.5
2
1.5
1
0.5
0
OD600 nm
0
1
2
3
4
5
6
7
8
9
10
11
12
Incubation time (h)
ATCC 25922 (TD 0 µg ml⁻¹)
NCV-4539 (TD 0 µg ml⁻¹)
SCV-4539 (TD 100 µg ml⁻¹)
SCV-4539 (TD 10 µg ml⁻¹)
SCV-4539 (TD 1 µg ml⁻¹)
SCV-4539 (TD 0.1 µg ml⁻¹)
SCV-4539 (TD 0 µg ml⁻¹)

Fig. 2

Table S1. Oligonucleotide primers used in PCR and sequencing

Target gene	Primer	Sequence (5'-3')	Reference
thyA	Ec thyA-F	TTCCATCCCGATGATTGTC	This study
	Ec thyA-R	AAGGYGTCTCGAAGAATTTAAC	
$bla_{\text{CTX-M-1}}$ group	CTX-M-1-F	GCTGTTGTTAGGAAGTGTGC	24
	CTX-M-1-R	CCATTGCCCGAGGTGAAG	
$bla_{\text{CTX-M-2}}$ group	CTX-M-2-F	ACGCTACCCCTGCTATTT	24
	CTX-M-2-R	CCTTTCCGCCTTCTGCTC	
$bla_{\text{CTX-M-9}}$ group	CTX-M-9-F	GCAGATAATACGCAGGTG	24
	CTX-M-9-R	CGGCGTGGTGGTGTCTCT	
bla_{TEM}	T1	CCGTGTCGCCCTTATTCC	25
	T2	AGGCACCTATCTCAGCGA	
bla_{SHV}	S1	ATTTGTCGCTTCTTTACTCGC	25
	S2	TTTATGGCGTTACCTTTGACC	
$bla_{\text{CTX-M}}$	CTX-M1A	CTTCCAGAATAAGGAATC	26
	CTX-M1B	CCGTTTCCGCTATTACAA	

Table S2. Minimum inhibitory concentrations (MICs) of antimicrobial agents for normal-colony variants (NCVs)

	MIC (μg/mL)					
	NCV-4474		NCV-4478		NCV-4539	
Antimicrobial agent	5% SB MHA	MHA	5% SB MHA	MHA	5% SB MHA	MHA
Ampicillin	12	12	> 256	> 256	> 256	> 256
Amoxicillin-clavulanate	8	8	12	12	8	12
Ampicillin-sulbactam	4	6	48	32	12	12
Cephalothin	24	32	> 256	> 256	> 256	> 256
Cefoxitin	12	16	4	4	4	6
Cefotaxime	0.19	0.19	48	48	12	12
Cefotaxime-clavulanate	0.19	0.19	0.094	0.125	0.19	0.094
Ceftazidime	1	1	3	3	2	2
Cefepime	0.094	0.19	8	8	6	8
Aztreonam	0.25	0.5	6	8	6	12
Imipenem	0.19	0.25	0.25	0.25	0.25	0.19
Gentamicin	2	2	192	> 256	1.5	2
Amikacin	8	12	3	4	6	6
Tetracycline	1.5	2	1.5	2	1.5	2
Ciprofloxacin	0.016	0.016	0.003	0.004	0.003	0.008

SB, Sheep Blood; MHA, Muller Hinton agar

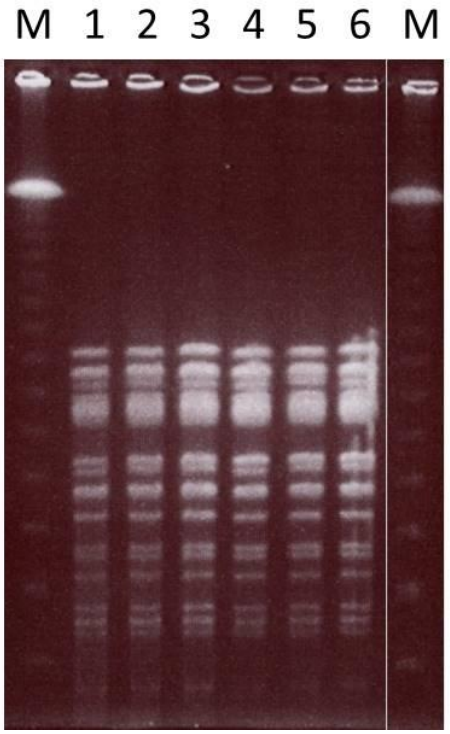

Fig. S1. Pulsed-field gel electrophoresis of the small-colony variants (SCVs) and normal-colony variants (NCVs)
Lanes: M, Marker; 1, SCV-4474; 2, SCV-4478; 3, SCV-4539; 4, NCV-4474; 5, NCV-4478; 6, NCV-4539

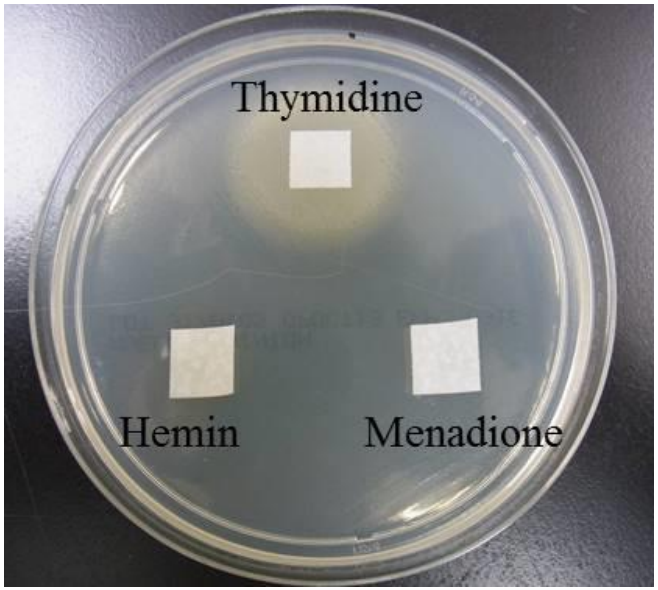

Fig. S2. Auxotrophy testing

Overnight incubation of SCV-4539 at 35°C on Mueller Hinton (MH) agar with each nutrient

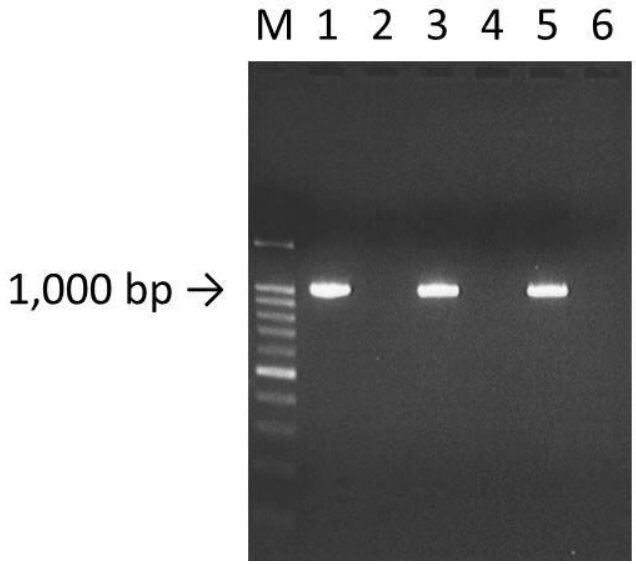

Fig. S3. *bla*$_{SHV}$ PCR of transconjugants

Lanes: M, Size marker; 1, *Klebsiella pneumoniae* ATCC700603; 2, SCV-4474; 3, Transconjugant in experiment (B); 4, *Escherichia coli* CSH2; 5, Transconjugant in experiment (C); 6, No template control

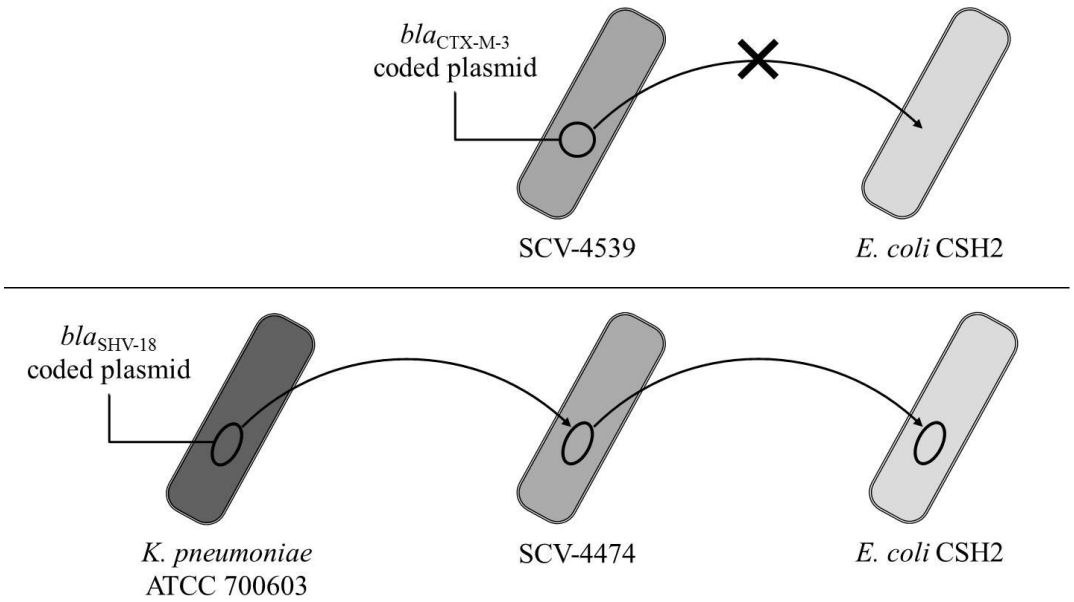

Fig. S4. Conjugational transfer of plasmids encoding ESBL genes