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Mycolic Acid-Containing Bacteria Induce Natural-Product Biosynthesis in *Streptomyces* Species▽†

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Natural products produced by microorganisms are important starting compounds for drug discovery. Secondary metabolites, including antibiotics, have been isolated from different *Streptomyces* species. The production of these metabolites depends on the culture conditions. Therefore, the development of a new culture method can facilitate the discovery of new natural products. Here, we show that mycolic acid-containing bacteria can influence the biosynthesis of cryptic natural products in *Streptomyces* species. The production of red pigment by *Streptomyces lividans* TK23 was induced by coculture with *Tsukamurella pulmonis* TP-B0596, which is a mycolic acid-containing bacterium. Only living cells induced this pigment production, which was not mediated by any substances. *T. pulmonis* could induce natural-product synthesis in other *Streptomyces* strains too: it altered natural-product biosynthesis in 88.4% of the *Streptomyces* strains isolated from soil. The other mycolic acid-containing bacteria, *Rhodococcus erythropolis* and *Corynebacterium glutamicum*, altered biosynthesis in 87.5 and 90.2% of the *Streptomyces* strains, respectively. The coculture broth of *T. pulmonis* and *Streptomyces endus* S-522 contained a novel antibiotic, which we named alchivemycin A. We concluded that the mycolic acid localized in the outer cell layer of the inducer bacterium influences secondary metabolism in *Streptomyces*, and this activity is a result of the direct interaction between the mycolic acid-containing bacteria and *Streptomyces*. We used these results to develop a new coculture method, called the combined-culture method, which facilitates the screening of natural products.

In modern microbiology, single-strain culture is the standard method for cultivating microorganisms. However, owing to the absence of interacting microorganisms that are present in the natural environment, the growth conditions in a flask culture are significantly different from those in the natural environment.

The members of the order *Actinomycetales*, especially the genus *Streptomyces*, produce a number of antibiotics and other bioactive natural products. The genomic analysis of some *Streptomyces* strains revealed the presence of biosynthetic gene clusters for about 30 secondary metabolites, and these data imply that a single *Streptomyces* strain can produce more than 30 secondary metabolites (1, 9, 12). However, some of these secondary-metabolite genes are not expressed in fermentation culture. To date, various methods (7, 14, 15, 17) have been used to activate genes synthesizing cryptic secondary metabolites. Secondary-metabolite production is affected by environmental factors (5, 17) such as temperature, the presence of hormone-like chemicals (6), and medium composition (3). Therefore, to identify new antibiotics, the isolation of new actinomycetes should be accompanied by the study of the effects of different growth conditions on each isolated strain.

Coculture is an effective method for inducing the production of cryptic metabolites. Some coculture methods have been reported (2, 18); however, these methods often are specific to two bacterial strains, because they are based on the specific mutual interaction between these strains. Therefore, it is difficult to extend these methods to other strains.

To overcome this limitation, we developed a novel fermentation method, the combined-culture method, involving the coculture of two bacterial strains. We found that *Streptomyces* and mycolic acid-containing bacteria interact with each other for inducing secondary metabolism, and we applied this method to the screening of natural products.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains used in this study are shown in Table 1. Strains were obtained from the Japan Collection of Microorganisms (JCM; RIKEN BioResource Center, Wako, Saitama, Japan) and NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan). Actinomycetes were isolated from plant tissues according to a previously described method (19). A-3M medium was used for the production of secondary metabolites, and V-22 medium was used for seed culture (14). Bennett's glucose agar, nutrient agar, and mannitol soya flour agar were used for actinomycetes isolation (14). A dialysis-culture flask was purchased from Ouchi Rikakogyo (Tokyo, Japan). A mixed cellulose ester dialysis membrane, ADVANTEC 0.2 μm, was purchased from Toyo Roshi Kaisya (Tokyo, Japan), and regenerated cellulose membrane, Spectra/Por 7 (pore size is about 50 kDa), was purchased from Funakoshi (Tokyo, Japan). For comparing the secondary-metabolite profiles of different combined cultures, we isolated 112 actinomycetes from soil samples collected in Hokuriku district, Japan. The analysis of the 16S rRNA genes of these isolated actinomycetes revealed that all of them were strains of *Streptomyces*. For antibiotic screening, another 97 actinomycetes were isolated from soil or plant tissue samples collected from Toyama Prefecture, Japan.

Screening of bacteria that induce red pigment production in *Streptomyces lividans*. To assay the induction activity, *S. lividans* spores were overlaid on Bennett's glucose agar plates. Each inducer bacterium then was inoculated on these plates, and the plates were incubated for 2 to 3 days at 30°C. The production of red pigments around the bacterial colonies was investigated. The spore overlay procedure has been described in our previous paper (14). Among the 400 bacteria used for the red pigment induction assay, 350 were obtained from the

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TABLE 1. Bacterial species used in this study and their activities in combined cultures

Bacterial species	Mycolic acid[a]	Inducing activity[b]	Red pigment production with[c]:	
			Act	Red
Streptomyces lividans TK23	−		0.2	ND
Tsukamurella pulmonis TP-B0596	+	+	1.9×10^2	11.0
Tsukamurella pseudospumae JCM13375^T	+	+	8.0	10.9
Tsukamurella spumae JCM12608^T	+	+	38.9	14.2
Tsukamurella strandjordii JCM11487^T	+	+	36.3	33.3
Corynebacterium glutamicum ATCC13869	+	+	11.6	38.2
Corynebacterium efficiens NBRC100395^T	+	+	5.7	3.0
Rhodococcus erythropolis JCM3201^T	+	+	15.8	34.7
Rhodococcus coprophilus JCM3200^T	+	+	31.0	14.1
Rhodococcus wratislaviensis JCM9689^T	+	+	3.3×10^2	5.0
Rhodococcus zopfii JCM9919^T	+	+	13.6	38.2
Gordonia rubripertincta JCM3204^T	+	+	80.4	11.9
Gordonia bronchialis NBRC16047^T	+	+	29.5	14.8
Nocardia farcinica JCM3088^T	+	+	1.1×10^2	7.8
Nocardia asteroides NBRC15531^T	+	+	11.1	1.5
Mycobacterium smegmatis NBRC3082	+	+	1.1×10^3	41.9
Mycobacterium chlorophenolicum NBRC15527^T	+	+	1.3×10^2	3.7
Williamsia muralis JCM10676^T	+	+	34.4	5.8
Dietzia cinnamea JCM13663^T	+	+	0.4	0.8
Dietzia maris JCM6166^T	+	−	ND	ND
Turicella otitidis JCM12146^T	−	−	0.6	ND
Pseudonocardia autotrophica JCM4348^T	−	−	0.6	ND
Bacillus subtilis ATCC 6633	−	−	ND	ND
Escherichia coli NIHJ JC-2	−	−	ND	ND
Staphylococcus aureus 209P JC-1	−	−	ND	ND
Micrococcus luteus TP-B100	−	−	ND	ND
Saccharomyces cerevisiae TP-F0176	−	−	ND	ND
Candida albicans TP-F0176	−	−	ND	ND

[a] Presence of mycolic acid in the outer layer.
[b] Inducing activity of red pigment production in *S. lividans*.
[c] The concentrations of actinorhodin (Act) and undecylprodigiosin (Red) are indicated as μM culture broth. ND, not detected.

actinomycete (TP-A) or bacterial (TP-B) culture library of Toyama Prefectural University, and 50 were newly isolated from soils collected in Toyama Prefecture, Japan.

Assay of red pigment-inducing activities of mycolic acid-containing bacteria. *S. lividans* TK23 and mycolic acid-containing bacteria were separately inoculated into test tubes containing 10 ml of V-22 medium. *S. lividans* was cultured at 30°C for 3 days on a rotary shaker at 200 rpm, and mycolic acid-containing bacteria were cultured at 30°C for 2 days on a rotary shaker at 200 rpm. Three ml of *S. lividans* culture and 1 ml of the culture of mycolic acid-containing bacteria were transferred into the same 500-ml K-1 flasks containing 100 ml of A-3M medium, and the resulting combined culture was incubated at 30°C for 7 days on a rotary shaker at 200 rpm. The amounts of actinorhodin and undecylprodigiosin were measured separately by the previously described procedures (10). For the measurement of actinorhodin, 1-ml aliquots of culture broths were treated with 200 μl of 6 N KOH (final concentration, 1 N) and vortexed thoroughly. The cell debris was removed by centrifugation ($3,000 \times g$ for 5 min), and the A_{640} values of the supernatants were measured ($e_{640} = 25{,}350\ M^{-1}\ cm^{-1}$) by using a DU640 spectrophotometer (Beckman Coulter).

For the measurement of undecylprodigiosin, 1-ml aliquots of the culture medium were harvested and centrifuged (15,000 rpm for 2 min), and the cell pellets were resuspended in 1 ml of methanol (pH 1.0). The samples were vortexed thoroughly and centrifuged (15,000 rpm for 2 min). The supernatants were collected and their A_{530} value was measured ($e_{530} = 100{,}150\ M^{-1}\ cm^{-1}$).

Preparation of mycolic acid and cell wall fraction and construction of a mycolic acid-deficient mutant strain. Mycolic acid was extracted according to previously described procedures (21). First, 100 ml of Luria-Bertani culture medium was harvested and centrifuged; the resulting wet cell pellet was resuspended in 20 ml of 10% KOH-MeOH and then hydrolyzed by exposure to 100°C for 2 h. The solution was cooled to room temperature, and the hydrolyzed residues were acidified with 6 N HCl and then extracted using 20 ml of *n*-hexane. The hexane phase was collected and evaporated *in vacuo*. An aliquot of the residue was resuspended in 20 ml of benzene-MeOH-H_2SO_4 (10:20:1) solution and then incubated for 2 h at 100°C. The solution was cooled to room temperature, and the methyl-esterified residue first was extracted using 20 ml of water and *n*-hexane. Mycolic acid was obtained by the concentration of the hexane phase. To confirm the extraction, an aliquot of the hexane phase was subjected to thin-layer chromatography (TLC) (silica gel 60 F_{254}; Merck); the TLC plates were developed in *n*-hexane-diethyl ether (4:1) solution and then soaked in 50% H_2SO_4. The plates were heated at 150°C, and the spots of methylester derivatives of mycolic acid were detected. The cell wall fraction of *Tsukamurella pulmonis* was extracted according to previously described procedures of the sonication-SDS method (20). The cell wall fraction yields approximately 10% of wet cell weight. In the additional experiments, mycolic acid prepared from the 100-ml culture was put into 10 ml of *S. lividans* pure culture, and the cell wall fraction prepared from the 500-ml culture was put into 10 and 100 ml of *S. lividans* pure cultures.

The mycolic acid-deficient mutant of *Corynebacterium glutamicum* was gifted by H. Kawasaki. The mycolic acid-deficient mutant was constructed according to the procedure described by Portevin et al. (16). Two independent mutants were gifted, and their red pigment-inducing activities were analyzed.

Analysis of secondary-metabolite profiles and antibiotic screening of the coculture broth. To assay the secondary metabolites, both combined and pure cultures were incubated at 30°C for 7 days on a rotary shaker at 200 rpm; subsequently, the entire culture broth was extracted with an equal volume of *n*-butanol, and the secondary metabolites in the extracts were assayed (a flowchart for the combined-culture procedure is shown in Fig. 3A). For secondary-metabolite profile analysis, the extracts were evaporated *in vacuo*, and the residue was dissolved in dimethyl sulfoxide and subjected to high-performance liquid chromatography (HPLC) analysis. HPLC analysis was performed using an HP 1090 system (Hewlett Packard) and a C_{18} Rainin Microsorb column (inner diameter, 4.6 mm; length, 100 mm; Rainin Instrument Co., MA). The temperature was set at 30°C and the flow rate at 1.2 ml/min. The solvent was composed of acetonitrile and an aqueous solution of 0.15% KH_2PO_4 (pH 3.5). The elution profile of secondary metabolites was monitored at 254 nm (see Fig. 4A). The differences in the antibiotic production of the cocultures and pure cultures were determined by studying the HPLC profile. The number of strains with altered secondary-metabolite production was counted, and these data are presented as Venn diagrams (see Fig. 3B to F). The antibacterial activity of each culture broth was measured by performing a paper disc diffusion assay on agar plates. The indicator bacteria were overlaid on nutrient agar plates, and paper discs immersed in the broth extracts then were placed on these plates. Antibacterial activity was estimated by measuring the diameter of the inhibitory zone around the disc.

Purification and structure determination of alchivemycin A. To purify alchivemycin A, we extracted 3 liters of the combined-culture broth (*Streptomyces endus* S-522 and *T. pulmonis*) with 1.5 liters of *n*-butanol. After the evaporation of *n*-butanol, the residue was dissolved in methanol and applied to a silica gel column (silica gel 60N; 63 to 210 μm; Kanto Chemical, Japan). The column was eluted with a stepwise gradient of chloroform-methanol (20:1 to ~0:1). The fractions containing antibiotic activity were eluted using chloroform-methanol (4:1 and 2:1). These fractions were pooled and evaporated to obtain crude alchivemycin A. The crude alchivemycin A was dissolved in dimethyl sulfoxide and applied to a reverse-phase silica gel column (ODS-AM; internal diameter, 200 by 46 mm; YMC, Japan). The column was eluted with a stepwise gradient of acetonitrile and 0.15% K_2HPO_4 (pH 3.5) (2:8 to 9:1). The fraction containing antibacterial activity (8:2) was evaporated *in vacuo*. The resulting aqueous layer was extracted with ethyl acetate. The organic layer was dried *in vacuo* to obtain pure alchivemycin A (25 mg).

^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained at 750 and 189 MHz in dimethyl sulfoxide-d_6. Liquid chromatography-mass spectrometry (LC-MS) spectra were obtained on an AP1165 machine (Applied Biosystems). UV-visible spectra were taken on an HP1090 system. NMR assignment data are shown in Table S1 and Fig. S7 in the supplemental material.

Nucleotide sequence accession numbers. The 16S rRNA sequences were submitted to the DDBJ database under accession numbers AB564290 (*Streptomyces endus* S-522) and AB564289 (*Tsukamurella pulmonis* TP-B0596).

RESULTS

Isolation of bacteria that induce secondary-metabolite production. In this study, we used the production of two red pigments by *Streptomyces lividans* TK23 under certain conditions as a indicator of secondary metabolism (10). In Bennett's glucose medium, *S. lividans* does not produce the two red

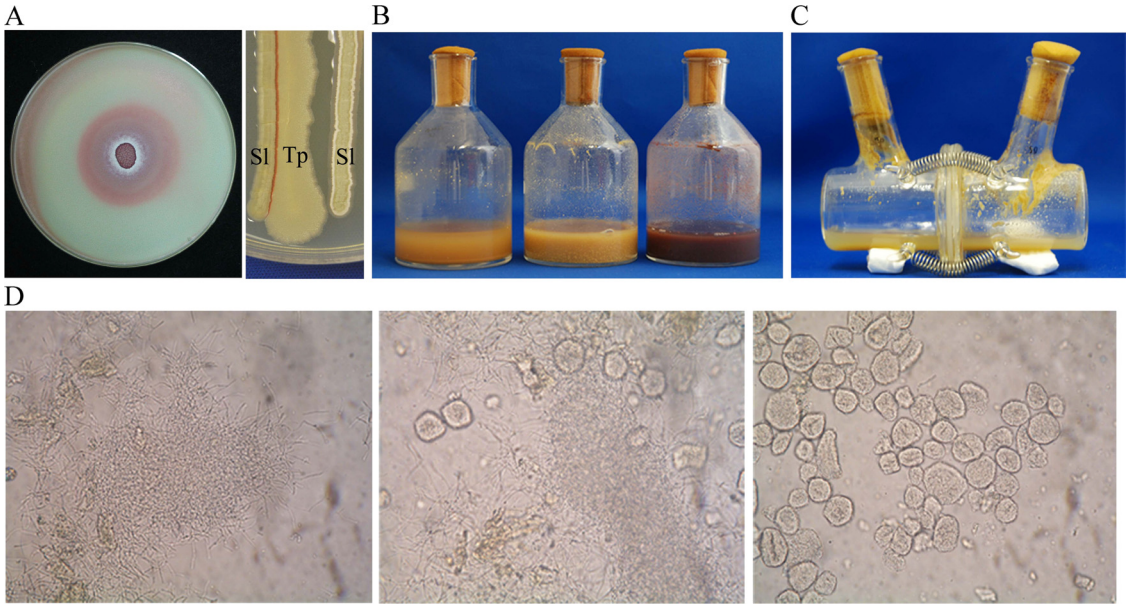

FIG. 1. Induction of secondary-metabolite production by mycolic acid-containing bacteria in a coculture with *S. lividans* TK23. The coculture of *T. pulmonis* and *S. lividans* was performed to induce the production of secondary metabolites. (A) Coculture of *T. pulmonis* and *S. lividans* in Bennett's medium. *S. lividans* spores were overlaid, and *T. pulmonis* was inoculated at the center of the plate (left). *S. lividans* and *T. pulmonis* were inoculated in parallel on the medium (right). Sl, *S. lividans*; Tp, *T. pulmonis*. Red pigment production was found only at the site of cell-to-cell contact. The organisms were grown at 30°C for 3 days. (B) Liquid medium without bacteria (left), pure culture of *S. lividans* (center), and coculture (right). The organisms were grown at 30°C for 3 days in A-3M medium. (C) Coculture of *T. pulmonis* and *S. lividans* in a dialysis flask. *T. pulmonis* was grown in the left compartment and *S. lividans* in the right compartment at 30°C for 7 days. Red pigments were not detected in the broth. (D) Optical micrograph of *S. lividans* (left), coculture (center), and *T. pulmonis* (right) at 1,000-fold magnification.

pigments actinorhodin (23) and undecylprodigiosin (4). We then screened bacterial strains that induce the production of these two pigments on Bennett's glucose plate. Of the 400 inducer strains assayed, 1 bacterial strain, TP-B0596, induced red pigment production (Fig. 1A), even in liquid cultures (Fig. 1B). Microscopic studies revealed that the TP-B0596 and TK23 strains partly intertwined in liquid culture (Fig. 1D). The scanning electron micrograph showed that TP-B0596 was a coryneform bacterium (see Fig. S1a in the supplemental material), and the phylogenetic analysis of the 16S rRNA genes revealed that it was *Tsukamurella pulmonis* (25).

Induction of red pigment production is not mediated by molecular substances but by cell-to-cell interactions. The liquid culture of *T. pulmonis* extracted with *n*-butanol or sterilized using a 0.2-μm filter did not induce pigment production. Moreover, liquid culture broth sterilized in an autoclave at 121°C for 1, 3, 10, or 30 min also did not show this inducing ability (see Fig. S1b in the supplemental material). These results imply that pigment induction was mediated by living cells and not by substances such as microbial hormones (6). We next investigated whether the inducing ability exists when *T. pulmonis* is cocultivated with *S. lividans* in a dialysis flask. A dialysis flask consists of two compartments partitioned with a dialysis membrane; this arrangement allows the exchange of small molecules between the compartments during microbial cultivation (13). A 0.2-μm mixed cellulose ester membrane and a regenerated cellulose membrane were used as the dialysis membranes for separation. *S. lividans* did not produce red pigments in the dialysis culture of the mixed cellulose ester membrane (Fig. 1C) and of the regenerated cellulose membrane (see Fig. S1c and d in the supplemental material), indicating that the pigment-inducing ability of *T. pulmonis* requires cell-to-cell interactions. On solid culture, cell-to-cell interaction also is needed to induce the pigments. *S. lividans* and *T. pulmonis* were inoculated next to one another (Fig. 1A). The red pigment was produced only in part of the cell-to-cell interaction.

Coryneform bacteria are potent inducers of secondary metabolism. Three closely related strains, *T. pseudospumae*, *T. spumae*, and *T. strandjordii*, also showed pigment-inducing ability (Table 1; also see Fig. S2a in the supplemental material). The genus *Tsukamurella* belongs to the family *Corynebacteriaceae*; the members of this family show the presence of mycolic acid in the outer layer of the cells. Therefore, we investigated the relationship between the red pigment induction and mycolic acid. First, we performed experiments using other mycolic acid-containing members of *Corynebacteriaceae* (the phylogenetic tree is shown in Fig. S3 in the supplemental material). Among the 19 species tested, those belonging to the genera *Rhodococcus*, *Corynebacterium*, *Nocardia*, *Mycobacterium*, *Williamsia*, *Dietzia*, and *Gordonia* induced pigment formation in *S. lividans* (Table 1). In contrast, the widely divergent species belonging to the genera *Pseudonocardia*, *Turicella*, *Escherichia*, *Staphylococcus*, *Bacillus*, *Micrococcus*, *Saccharomyces*, and *Candida*, which do not possess mycolic acid in the outer layer of the cell, did not induce pigment production (Table 1; also see Fig. S2b in the supplemental material).

Mycolic acid is responsible for inducing red pigment production. To determine whether mycolic acid is responsible for the pigment-inducing activity, we used a *C. glutamicum* strain with a disrupted mycolic acid biosynthetic gene, *pks13*, as a

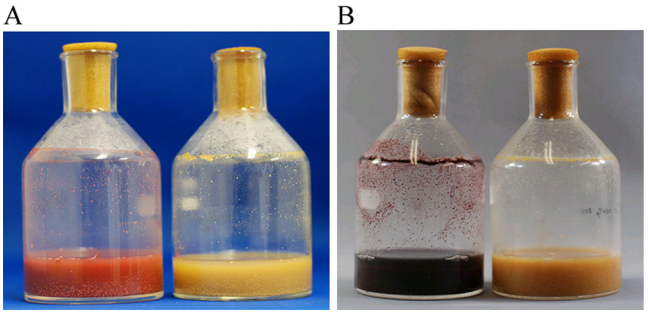

FIG. 2. Effect of mycolic acid on the induction of secondary metabolites in the coculture. (A) Coculture of *S. lividans* with wild-type *C. glutamicum* (left) and with Δ*pks13*, the mycolic acid-deficient mutant of *C. glutamicum* (right). In this experiment, two Δ*pks13* mutants were used independently for coculture. (B) Effect of isoniazid on metabolite production. Shown are the coculture of *S. lividans* with *T. pulmonis* (left) and coculture with added isoniazid (right).

mycolic acid-deficient mutant; the absence of mycolic acid in this mutant was confirmed (see Fig. S4 in the supplemental material), and the mutant had no significant physiological differences from its parental strain. In a coculture with *S. lividans*, the disruptant did not induce red pigment production (Fig. 2A). We then studied the effect of an inhibitor of mycolic acid biosynthesis on red pigment production. Isoniazid is an inhibitor of mycolic acid synthase (22) and has no effect on the growth of *S. lividans* at less than 100 mM (11). The addition of isoniazid to the coculture of *S. lividans* and *T. pulmonis* inhibited red pigment production (Fig. 2B). Similar results were obtained when isoniazid was added to the cocultures of *S. lividans* and other mycolic acid-containing species, like *Rhodococcus*, *Corynebacterium*, *Gordonia*, and *Nocardia* (*Rhodococcus erythropolis* and *Corynebacterium glutamicum*; see Fig. S5a in the supplemental material). Actinorhodin and undecylprodigiosin are polyketide compounds, and the possibility of their biosynthesis inhibition by isoniazid was examined by using *Streptomyces coelicolor* A3(2), which is another actinorhodin and undecylprodigiosin producer. At the concentration of 7.2 mM isoniazid, *S. coelicolor* A3(2) can produce actinorhodin and undecylprodigiosin (data not shown). This result suggests that isoniazid does not affect the actinorhodin and undecylprodigiosin biosynthetic enzymes, and the decrease in mycolic acid by isoniazid directs the inactivation of the biosynthesis.

These results indicate that mycolic acid is required for inducing red pigment production in *S. lividans*. However, the addition of mycolic acid extract obtained from *C. glutamicum*, *T. pulmonis*, *T. spumae*, *Rhodococcus zopfii*, and *Rhodococcus wratislaviensis* did not induce red pigment production in *S. lividans* (see *C. glutamicum* in Fig. S5b in the supplemental material), and whole-cell-wall fraction extracted from *T. pulmonis* also did not induce red pigment production in *S. lividans* (see Fig. S1e in the supplemental material). These results suggest that the mycolic acid present in the outer layer is important for inducing red pigment production, and the outer layer itself influences the inducing activity.

Induction of secondary-metabolite production varies with each strain. We identified the pigments (actinorhodin or undecylprodigiosin) induced and their concentrations in the coculture broths of each inducer strain and *S. lividans* (Table 1). Each strain induced a significantly different type and concentration of red pigments. While coculture with *T. pulmonis* primarily yielded actinorhodin, coculture with *C. glutamicum* yielded undecylprodigiosin. We then determined whether the pH of each coculture broth was different from that of the pure-culture broth (see Fig. S6 in the supplemental material). The pH values of the cocultures with mycolic acid-containing strains varied from 4.6 to 7.5.

Secondary metabolism in other *Streptomyces* strains is influenced by mycolic acid-containing bacteria. We determined whether *T. pulmonis* influences the secondary metabolism of not only *S. lividans* but also other *Streptomyces* strains. For this, we compared the secondary-metabolite profiles, determined using HPLC, between the cocultures and the pure cultures. A total of 112 new *Streptomyces* strains were isolated for this experiment. The corresponding HPLC peaks were compared between the pure cultures and cocultures; on the basis of this comparison, the HPLC peaks were grouped into the following four basic regulation patterns: increase/decrease in metabolite production, appearance of new metabolites, disappearance of some secondary metabolites, and no difference. These four patterns were then subdivided into eight combinations in each *Streptomyces* strain, because the strains did not uniformly produce secondary metabolites. For example, in some strains, we found that one metabolite was produced in a greater quantity in coculture than in pure culture, whereas another metabolite was produced in lesser quantity in coculture than in pure culture (Venn diagrams are shown in Fig. 3B to F). In the first regulation pattern, coculture induced an increase or decrease in the height of an existing peak. Coculture with *T. pulmonis* increased secondary-metabolite production in 61 strains and decreased production in 49 strains (Fig. 3B). In the second regulation pattern, coculture induced the appearance of a new metabolite peak or the disappearance of an existing peak. Of 112 strains, *T. pulmonis* induced the production of new secondary metabolites in 41 strains and suppressed secondary metabolite production in 12 strains. In the third pattern, which was observed in 13 strains, there were no differences between the profiles of the pure cultures and cocultures with *T. pulmonis*. A summary of the data described above reveals that *T. pulmonis* induced the production of new secondary metabolites in 36.6% of strains (41/112) and increased metabolite production in 54.5% of strains (61/112) (Fig. 3B). These results suggest that *T. pulmonis* can induce secondary-metabolite production in many diverse *Streptomyces* species. *R. erythropolis* (Fig. 3C) and *C. glutamicum* (Fig. 3D) showed the same ability, and they induced the production of new secondary metabolites in 36 and 27 *Streptomyces* strains, respectively (Fig. 3F). The HPLC profiles showed that some metabolites were induced by all three inducer strains, whereas others were induced by only one or two inducer strains (Fig. 3E and F). These results are consistent with the finding that each mycolic acid-containing strain induces the production of different concentrations of red pigments by *S. lividans*, and we named our coculture procedure combined culture.

Discovery of a new antibiotic, alchivemycin A, by combined-culture screening. We then used the combined culture for antibiotic screening. We isolated 97 new actinomycetes and analyzed the extracts of the combined cultures by using antibiotic assays against six microorganisms. The results of these

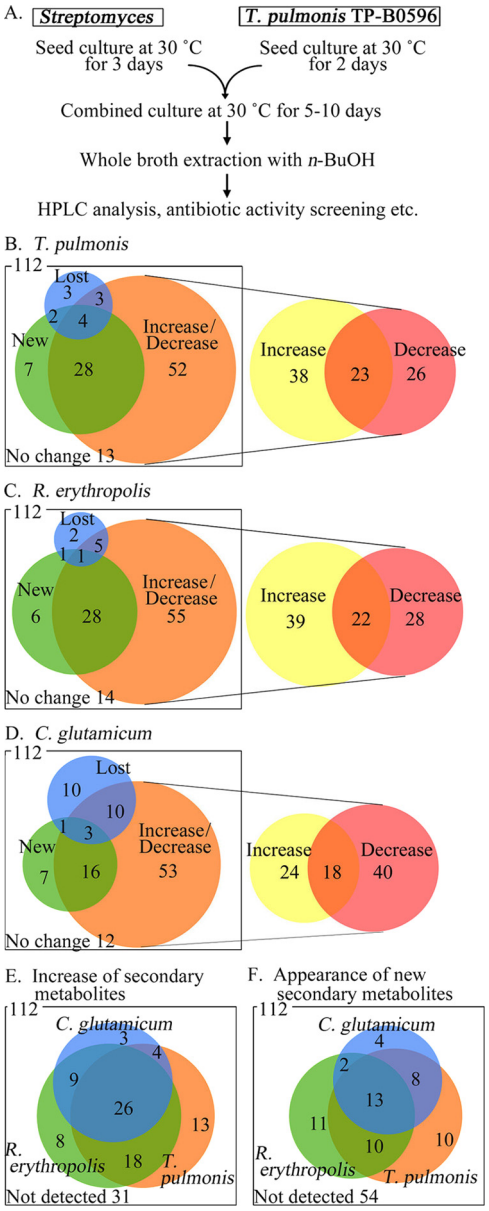

FIG. 3. Effects of the coculture of *Streptomyces* isolated from soil. Metabolite profiles of pure and combined cultures of *Streptomyces*. (A) Flowchart of the coculture procedure. (B to F) The results of the comparison between the secondary-metabolite profiles of the pure and cocultures are shown in a Venn diagram. (B to D) Comparison between the HPLC profiles of a pure culture of *Streptomyces* and cocultures. Each profile was classified according to the increase/decrease in metabolite production, appearance of new metabolites, and disappearance of some secondary metabolites. The increase/decrease part is subclassified into increase, both increase and decrease, and decrease in the right panel. (E and F) Comparison of the HPLC profiles of cocultures of *Streptomyces* with *T. pulmonis*, *R. erythropolis*, or *C. glutamicum*. The numerals represent the number of strains. New, new secondary metabolites appeared in the coculture; increase/decrease, the secondary-metabolite levels in the coculture were higher/lower than the levels in the pure culture; lost, secondary-metabolite production was absent in the coculture; no change, there was no difference between the secondary-metabolite levels in the pure culture and cocultures.

TABLE 2. Paper disc diffusion assays for antibacterial activities of different actinomycete culture broths[a]

Bacterial strain	*T. pulmonis*	*E. coli*	*B. subtilis*	*S. aureus*	*M. luteus*	*C. albicans*	*S. cerevisiae*
S. lividans TK23	+	10	10				
	−						
S. coelicolor A3(2)	+	11	12				
	−						
S501	−					19	22
	−						
S510	+	23		22			
	−						
S522	+			40			
	−						
S536	+		16	12	14		
	−						
S558	+	11	17	15	19	17	18
	−						
S566	+		19	16	22	16	20
	−						
S573	+		25	23	20		
	−		15	13	12		
S576	+		16	15	13		
	−				20		
S589	+				15		
	−						

[a] Actinomycetes isolated from the soil and plant tissues were cultured as pure cultures (−) or combined cultures with *T. pulmonis* (+). The culture medium was extracted using *n*-butanol and assayed by a paper disc method (diameter of the disc, 10 mm). The numbers represent the diameter of the inhibitory zones (mm).

assays were compared to the corresponding results for pure cultures. *T. pulmonis* induced or activated antibiotic production in 11 of the 97 strains studied (Table 2). In 10 of these 11 strains, the antibiotic production was initiated only in combined culture, and antibiotic activity was increased in the remaining strain. We then identified the antibiotics induced by combined culture. In a combined culture with *T. pulmonis*, S-522 showed specific antibiotic activity against *Micrococcus luteus*, whereas the S-522 or *T. pulmonis* pure cultures did not show this activity (Table 2). We studied the HPLC profiles of the *n*-butanol extracts of S-522 cultures that were incubated with *T. pulmonis* (Fig. 4A) or *C. glutamicum* (Fig. 4B) or without *T. pulmonis* (Fig. 4C).

Strain S-522 was isolated from a leaf of *Allium tuberosum*, and the phylogenetic analysis of the 16S rRNA genes identified it as *Streptomyces endus* S-522. The HPLC profiles showed that the secondary-metabolite pattern of the combined culture was different from that of the pure S-522 culture. The antibiotic secreted by this strain was identified as the peak that eluted at 23 min (Fig. 4A and B). After purification, 25 mg of pure antibiotic was obtained from 3 liters of combined-culture broth. The chemical structure of the antibiotic was studied using NMR and MS (see Table S1 and Fig. S7 in the supplemental material; the absolute configuration will be described in another paper). On the basis of these analyses, we concluded that this antibiotic has a novel chemical structure and named it alchivemycin A (Fig. 4E). The molecular formula and mass-to-charge ratio (*m/z*) of alchivemycin A are $C_{35}H_{53}NO_{10}$ and 648.3754 $[M+H]^+$, respectively. This antibiotic contains a heterocyclic chromophore that has not been described previously

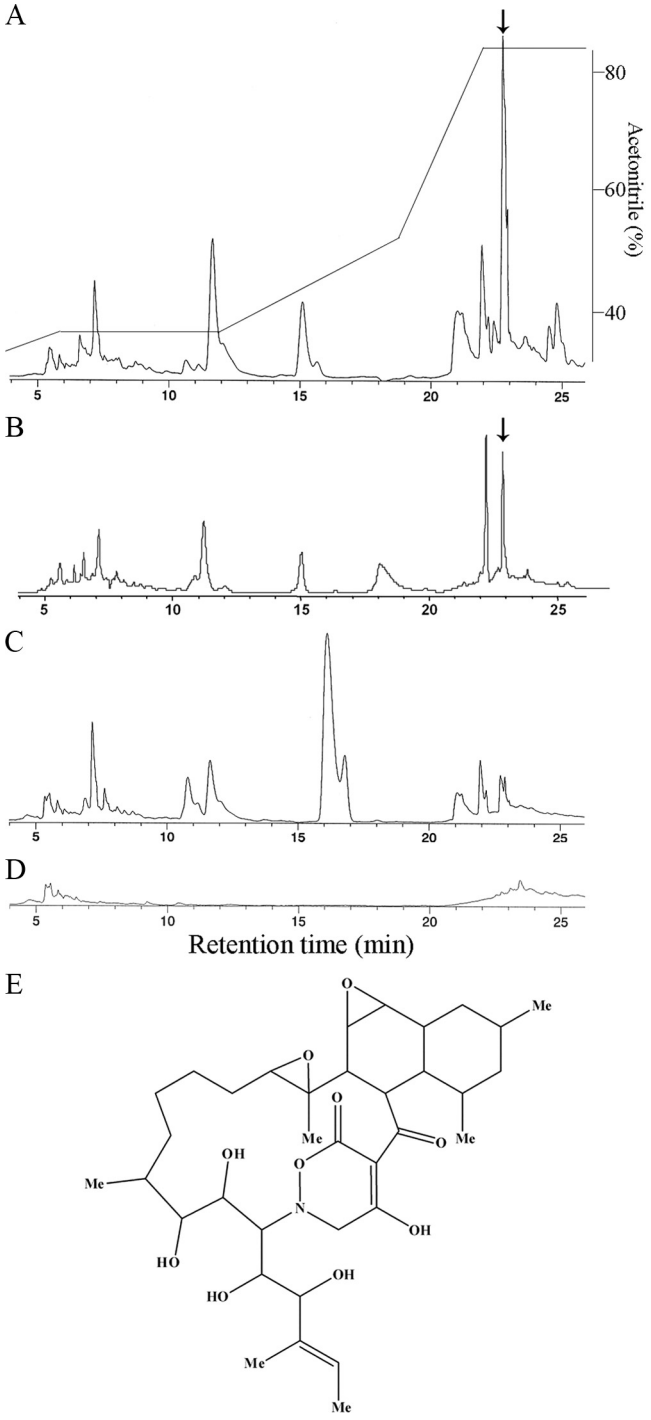

FIG. 4. Production of a novel antibiotic, alchivemycin A, in a combined culture of *Streptomyces endus* S-522 and *T. pulmonis* or *C. glutamicum.* Shown is the production of alchivemycin A in a combined culture of *S. endus* S-522 and *T. pulmonis* or *C. glutamicum*. (A to D) HPLC profiles of the secondary metabolites produced by S-522 cultured with or without *T. pulmonis*. Shown are the culture broths of the combined culture with *T. pulmonis* (A), combined culture with *C. glutamicum* (B), S-522 pure culture (C), and *T. pulmonis* pure culture (D). The arrow shows the peak for alchivemycin A. Elution was performed with a linear gradient, as indicated on the scale at the right. (E) Chemical structure of alchivemycin A.

TABLE 3. Antibacterial activities of alchivemycin A[a]

Organism	Relevant characteristic	Alchivemycin A MIC (μg/ml)
Micrococcus luteus	Gram positive	0.06
Bacillus subtilis	Gram positive	40
Staphylococcus aureus	Gram positive	>50
Escherichia coli	Gram negative	>50
Saccharomyces cerevisiae	Yeast cell	>50
Candida albicans	Yeast cell	>50

[a] The MICs were determined by the 2-fold sequential dilution method in heart infusion broth (Difco). The inhibition concentration was determined by measuring the absorbance at 600 nm.

and shows specific antibiotic activity against *Micrococcus luteus* at a concentration of 0.06 μg/ml (Table 3).

DISCUSSION

In this study, we found that *T. pulmonis* TP-B0596 induces the secondary metabolism of different *Streptomyces* species. Further analysis revealed that other species belonging to the *Tsukamurella* genus and related genera have similar activity against *Streptomyces*. Some substances, such as the γ-butyrolactone autoregulators (6), desferrioxamine E (24), and goadsporin (14), have been reported to induce secondary metabolism in *Streptomyces*. Dialysis cultures and the addition of some components prepared from the mycolic acid-containing bacteria to *Streptomyces* pure culture revealed that molecular substances do not mediate this inducing ability. Therefore, the induction mechanism in a combined culture is different from substance-mediated induction. In a liquid culture, *Streptomyces* grows in filaments and *T. pulmonis* aggregates to pellet form. In a solid culture, red pigment production was found only at the site of cell-to-cell contact. These results indicate that the inducer bacteria and *Streptomyces* intertwine in the combined culture, and the inducer bacteria form cell-to-cell interactions with *Streptomyces* via mycolic acid and therefore influence the secondary metabolism.

The mycolic acid-containing members of *Corynebacteriaceae* induced pigment formation in *S. lividans*. In contrast, a wide variety of mycolic acid-deficient bacteria, including the *C. glutamicum* mycolic acid-deficient mutants, cannot induce pigment production. Mycolic acids are located in the outer layer of the bacterium belonging to *Corynebacteriaceae*. They are bound to arabinogalactan, trehalose, and proteins, and they are important components of the highly impermeable outer barrier (8). However, the addition of purified mycolic acids into the pure culture of *S. lividans* had no effect on red pigment production. These results revealed that mycolic acid alone is not sufficient and the intact cells are required for the induction. We therefore conclude that the mycolic acid that exists in the outer layer is important for inducing red pigment production, and the outer layer itself influences the inducing activity.

Each mycolic acid-containing strain induces different changes in secondary metabolism. In a combined culture, we find that the combined culture broth of each inducer strain had a different pH. However, there is no relationship between pH values and red pigment productivity.

On the basis of our observations, the main factors affecting

the red pigment induction mechanism in the combined culture are (i) the direct cell-to-cell interactions between *Streptomyces* and mycolic acid-containing bacteria, and (ii) the changes in medium composition due to the primary metabolism of each inducer bacterium.

The changes in the combined culture reflect the changes in secondary metabolism induced by environmental conditions. Ten of the 18 mycolic acid-containing bacteria having the ability to induce secondary metabolism in *Streptomyces* were isolated from the soil samples (see Table S2 in the supplemental material). In the natural environment, these mycolic acid-containing bacteria may influence secondary metabolism in *Streptomyces*, which is one of the major inhabitant strains of soil. The determination of the molecular changes occurring in combined cultures will elucidate not only the process of the induction of secondary-metabolite production but also the environmental bacterial interactions.

We used our combined-culture method for screening bioactive compounds. The combined culture of *S. endus* S-522 and *T. pulmonis* yielded a novel antibiotic, alchivemycin A. We concluded that alchivemycin is produced by *S. endus*, with the following two reasons: (i) the production of these metabolites also was detected in the coculture of *S. endus* and *C. glutamicum*, and (ii) we did not detect alchivemycin in the coculture containing the other *Streptomyces* strains and *T. pulmonis*.

Combined culture is an easy method for inducing the production of cryptic antibiotics, because it only involves the addition of a mycolic acid-containing bacterium to a pure culture of an actinomycete. We believe that our combined-culture method will prove very useful for the screening of natural products.

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