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ORIGINAL ARTICLE

Rare earth elements activate the secondary metabolite–biosynthetic gene clusters in *Streptomyces coelicolor* A3(2)

Yukinori Tanaka[1,2], Takeshi Hosaka[3] and Kozo Ochi[1,4]

Genome sequencing projects have revealed many biosynthesis gene clusters for the production of as-yet unknown secondary metabolites, especially in actinomycetes. Here, we report that the rare earth elements, scandium and/or lanthanum, markedly activate, ranging from 2.5- to 12-fold, the expression of nine genes belonging to nine secondary metabolite–biosynthetic gene clusters of *Streptomyces coelicolor* A3(2) when added to the medium at low concentrations. HPLC analysis of ethyl acetate-extractable metabolites indicated the detectability of several compounds only in the rare earth-treated cultures. This approach should facilitate discovery of new biologically active compounds and the study of secondary metabolite production.
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Keywords: lanthanum; rare earth; scandium; silent genes; *Streptomyces coelicolor*

INTRODUCTION

Actinomycetes produce a variety of natural products that are of major importance in the pharmaceutical industry. More than 50% of all anti-infective and anticancer compounds developed over the past 25 years have been natural products or derivatives of such products.[1] There is accumulating evidence that the ability of actinomycetes to produce antibiotics and other bioactive secondary metabolites has been underestimated due to the presence of cryptic gene clusters. That is, genome sequencing projects have revealed many biosynthetic gene clusters for the production of unknown secondary metabolites. For example, *Streptomyces coelicolor*, *S. avermitilis*, *S. griseus* and *Saccharopolyspora erythraea* are each known to produce 3–5 secondary metabolites but actually possess >20 clusters that encode known or predicted biosynthetic pathways for secondary metabolites.[2–5] Exploitation of such genetic potential in actinomycetes may therefore lead to the isolation of new biologically active compounds.[6] Our laboratory has previously developed a method to increase antibacterial production.[7–9] This new approach, called 'ribosome engineering',[10,11] has several advantages. In this method, bacteria are grown on antibiotics to select antibiotic-resistant strains. Strains with mutations in the *rpsL* gene (encoding the ribosomal protein S12) or *rpoB* gene (encoding the RNA polymerase β-subunit) are isolated using streptomycin and rifampicin, respectively. These studies showed that mutations in ribosomal proteins and/or RNA polymerase alter bacterial gene expression, eventually leading to the discovery of novel antibiotics.[12] More recently, during the course of studying the effects of rare earth elements on bacterial physiology, we found that rare earths can elicit bacterial capabilities, and thus exert marked effects on secondary metabolism in streptomycetes, the typical soil microorganisms that often produce antibiotics.[13]

Rare earth is a general term for 17 elements that include scandium (Sc), yttrium (Y) and the lanthanides (15 elements from lanthanum (La) to lutetium (Lu)). Of the 17 elements, promethium (Pm) scarcely exists in universe as a radioisotope. Rare earth elements have been widely used in high-technology products, such as permanent magnets, fluorescent materials and new ceramics, and they are currently being used in computers, mobile telephones, plasma displays, magneto-optical disks, high-powered lasers, fluorescent lamps and hybrid cars.[14] Despite their importance in physics and chemistry, the significance of rare earths in biology has largely been overlooked. Working with *S. coelicolor* A3(2), the genetically best-characterized strain of *Streptomyces*, we report here that both scandium and lanthanum activate many secondary metabolite–biosynthetic genes, the majority of which may represent 'silent' or 'poorly expressed' genes in this organism.

MATERIALS AND METHODS

Bacterial strains and culture conditions

S. coelicolor A3(2) strain 1147, a prototrophic wild-type strain, which produces actinorhodin,[15] was used in this study. Cultivation was performed at 30 °C. GYM medium was described previously.[16] Scandium chloride hexahydrate ($ScCl_3 \cdot 6H_2O$; purity, 99.9%) and lanthanum chloride heptahydrate

[1]National Food Research Institute, Tsukuba, Ibaraki, Japan; [2]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka, Japan; [3]International Young Researchers Empowerment Center, Shinshu University, Nagano, Japan and [4]Hiroshima Institute of Technology, Department of Health Science, Faculty of Applied Information Science, Hiroshima, Japan
Correspondence: Dr K Ochi, Hiroshima Institute of Technology, Department of Health Science, Faculty of Applied Information Science, Miyake 2-1-1, Saeki-ku, Hiroshima 731-5193, Japan.
E-mail: k.ochi.bz@it-hiroshima.ac.jp
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($LaCl_3 \cdot 7H_2O$; purity, 99.9%), together with other rare earths (all chloride-salts; purity, >97%) were purchased from Wako Pure Chemical (Osaka, Japan). These rare earths were added to the autoclaved agar medium after cooling to 50–60 °C.

Transcriptional analysis by real-time quantitative PCR

Total RNAs were purified from cells grown on GYM plates covered with cellophane for the indicated times using Isogen reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. After treatment with RNase-free DNase I (amplification grade; Invitrogen, Carlsbad, CA, USA), 1 μg of each of the total RNAs was used as a template for reverse transcription (20 μl) with a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). The samples were diluted with an appropriate volume of water and analyzed using the 7300 real-time quantitative PCR (qPCR) system and THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan). Each transcriptional assay was normalized to the corresponding transcriptional level of the *hrdB* gene encoding the principal sigma factor. Primers used for real-time qPCR are listed in Table 1. All reactions were performed under the following conditions: 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C for denaturation and 40 s at 60 °C for annealing and extension.

Table 1 Primers used for real-time PCR

Gene	*Primer*	*Oligonucleotide sequence (5′→3′)*
hrdB	hrdB-F918	GGGCAACCTCGGTCTGATC
	hrdB-R980	GAGAACTTGTAGCCCTTGGTGTAGT
SCO0124	SCO0124-F1132	GAGGACCCGTCGGCATTG
	SCO0124-R1195	GGGTGAGGTAGGCCGTGAT
SCO0381	SCO0381-F1280	GCCCGGACATCCGAAGAC
	SCO0381-R1351	CGCTGCGTCCGCTGATCT
SCO0489	SCO0489-F3	GAGCACCAACCCCTTCGA
	SCO0489-R66	CTGGCCCTCGTCGTTCAC
SCO1207	SCO1207-F495	CACCGACCGGCACTCCAT
	SCO1207-R601	CCGAGAAGTAGGCGTTCATCTC
SCO1268	SCO1268-F544	GTCGGACAGGCGGAGGAA
	SCO1268-R609	GGGCAGGGAGACGAAACTG
SCO2785	SCO2785-F819	CCTGGCCCAGCAGTCCAT
	SCO2785-R889	GGGCAGTCTTCACGTAGTGCTT
SCO3215	SCO3215-F303	CGGACTGGTGCGCAAGGT
	SCO3215-R364	CGCAGGTGAGGATGTTGAAGT
SCO5085	actII-ORF4-F17	TGGGACGTGTCCATGTAATCA
(*act*II-ORF4)	actII-ORF4-R76	CCTTCGAGGATTTAAGCGGAAT
SCO5223	SCO5223-F814	CTCACCCCGGGCAGTGAA
	SCO5223-R866	GCCTGGAGCAACCACATGA
SCO5800	SCO5800-F1651	GACGAGCGCTTCGCCTACTA
	SCO5800-R1705	TGCCGATGAGACCGAACA
SCO5877	redD-F201	CGGACCCAGCCTGTACAACT
(*redD*)	redD-R265	CGATCGATACGGGTCCCAAT
SCO6283	SCO6283-F312	CACGAGCGAGGCCTTCCT
	SCO6283-R406	CGAAGTTCTGCGCGAACCA
SCO6430	SCO6430-F458	TGCAGTCCACCCAGATGTTC
	SCO6430-R579	CCAGACGGTGACCACGTACA
SCO6766	SCO6766-F35	CTACATACCTGGCCGAACAGAAG
	SCO6766-R91	CCACGATGAGCGGGAACT
SCO6826	SCO6826-F772	AGGGTCTGCCACGTGTTCA
	SCO6826-R827	GGGTCGAGGATGACCTTCAG
SCO7670	SCO7670-F278	TCGGGCCCTACTGGAACAC
	SCO7670-R382	CCACGACCGCGAGGTAGTT
SCO7684	SCO7684-F629	ACACCGAACACCGGTCCTT
	SCO7684-R748	CGGGATGGACGTTGTACCA

HPLC analysis of culture extracts

Spores and hyphal fragments of *S. coelicolor* 1147 were inoculated onto GYM agar medium (25 ml per culture plate (φ90×15 mm)) with or without scandium (200 μM) or lanthanum (1900 μM) and then incubated at 30 °C for 3 or 5 days. The agar medium with cells (from two plates) was extracted twice with 50 ml of EtOAc by gently agitating for 6 h. The EtOAc layer was collected and evaporated to dryness. The dried crude extract was dissolved in 5 ml of 50% aqueous MeOH, filtered through Cosmonice filter W (pore size, 0.45 μm; Millipore, Billerica, MA, USA), and subjected to HPLC. The HPLC apparatus consisted of a Shimadzu Prominence HPLC system equipped with diode array detector SPD-M20A (Shimadzu, Kyoto, Japan). Aliquots of 200 μl of each sample were loaded onto a column (CAPCELL PAK C18 MG II, 4.6×250 mm, 5 μm particle size; Shiseido, Tokyo, Japan) with a precolumn (CAPCELL PAK C18 MG II, 4.6×10 mm, 5 μm particle size; Shiseido) and eluted at a flow rate of 1 ml min^{-1}. Solvents and conditions used were as follows: 0–5 min, 40% CH_3CN containing 0.05% TFA; 5–40 min; a linear gradient from 40 to 100% CH_3CN containing 0.05% TFA; 40–50 min; 100% CH_3CN containing 0.05%

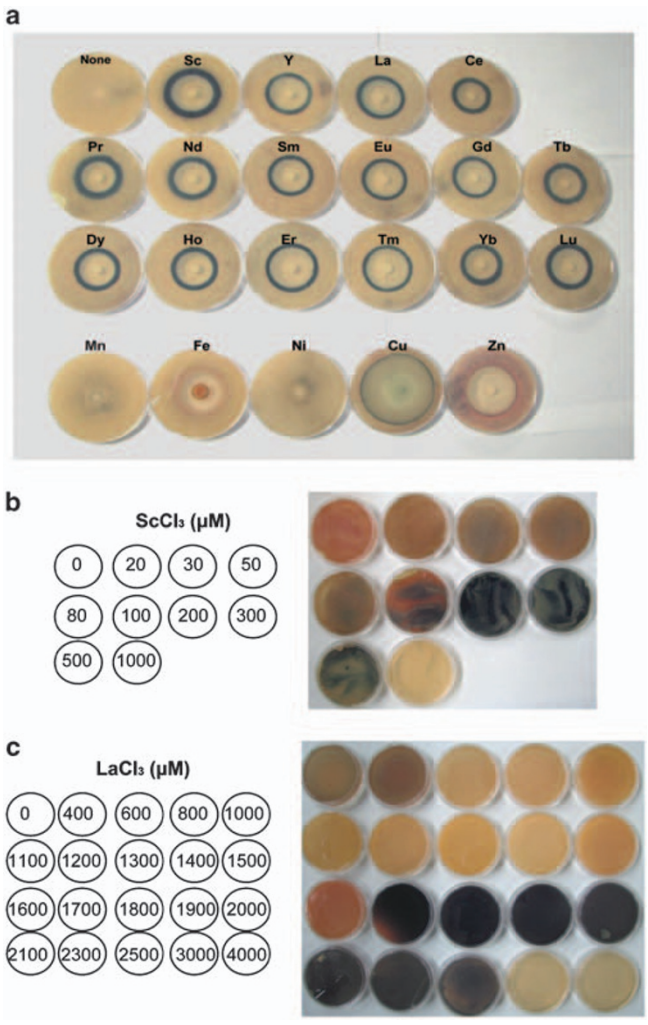

Figure 1 Effects of exogenously added rare earths on actinorhodin production. (**a**) *S. coelicolor* wild-type strain 1147 was spread on GYM agar, and a paper disk containing 4 mg of each rare earth (all chloride salts) was placed in the center of the plate. The plates were incubated at 30 °C for 4 days. Blue represents the antibiotic actinorhodin, whereas the clear zone of the center represents growth inhibition. (**b**, **c**) *S. coelicolor* 1147 was spread on GYM agar containing various amounts of scandium or lanthanum, followed by incubation for 4 days. Complete growth inhibition was detected at 1200 μM scandium and 5000 μM lanthanum.

TFA. Multiple wavelength monitoring was performed at 215, 254, 320, 450, 530 and 640 nm.

RESULTS AND DISCUSSION

Rare earths enhance actinorhodin production by *S. coelicolor*

Previously, we showed that several rare earths, including scandium and lanthanum, activate actinorhodin production in *S. coelicolor* and *S. lividans* when added to solid or liquid medium.[13] Therefore, we first examined the efficacy of all (except for promethium (Pm)) rare earths—scandium (Sc), yttrium (Y), lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu) (all chloride salts)—in enhancing actinorhodin production by *S. coelicolor*. As shown in Figure 1a, these rare earths were all effective in enhancing actinorhodin production, and the effect of scandium was most pronounced. In contrast, manganese (Mn), iron (Fe), nickel (Ni), copper (Cu) and zinc (Zn) (all chloride salts) were not effective. Scandium was effective at concentrations of 100–500 μM, with the optimal concentration of 200 μM, determined using plates containing fixed concentrations of scandium (Figure 1b). Conversely, lanthanum was effective over a limited concentration range of 1700–2500 μM, with the optimal concentration of 1900 μM (Figure 1c).

Effects of scandium and lanthanum on transcription of secondary metabolite–biosynthetic gene clusters

A recent study in our laboratory indicated that certain mutations in *rpoB* (encoding RNA polymerase β-subunit) or *rpsL* genes can activate 'silent' genes of actinomycetes or *Bacillus subtilis*, leading to the discovery of novel antibacterial agents.[12,17] The activation of silent genes by generating *rpoB* H437D or *rpoB* H437L mutations in *Streptomyces* sp. 631689 was attributed, at least in part, to the increased affinity of the mutant RNA polymerase for promoters. Later, we found that *rsmG* mutations, which confer a low level of resistance to streptomycin, can activate not only streptomycin production but also the expression of other secondary metabolite–biosynthetic genes in *S. griseus*, although the effects were not dramatic.[18] Therefore, our next interest was to study the effects of rare earths on the expression of secondary metabolite–biosynthetic gene clusters present in the *S. coelicolor* wild-type strain. A total of 17 genes belonging to 17 secondary metabolite–biosynthetic gene clusters (Table 2) were subjected to transcriptional analysis by real-time qPCR, using the cells grown with the optimal concentrations of scandium and lanthanum (200 and 1900 μM, respectively). Analysis of cells harvested at late growth phase (36, 48 and 60 h) indicated that scandium and lanthanum both markedly activated not only the actinorhodin biosynthetic gene cluster (as represented by *act*II-ORF4) but also the expression of other secondary metabolite–biosynthetic genes (Figure 2). The profiles of changes in expression of genes belonging to each secondary metabolite–biosynthetic gene cluster are shown in Supplementary Figure S1. Marked enhancement of expression was observed in *act*II-ORF4 (12-fold), SCO0489 (4-fold),

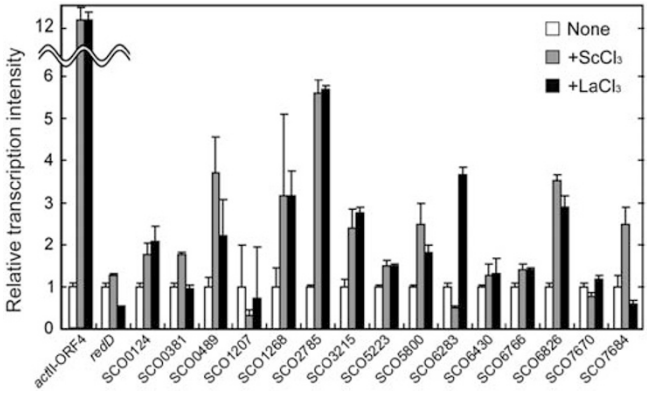

Figure 2 Transcriptional analysis of the genes involved in the secondary metabolite–biosynthetic gene clusters. The RNAs were extracted from cells grown to late growth phase (36, 48 and 60 h). Total RNA preparation and real-time qPCR was performed as described in Materials and methods section. The maximum expression levels were compared, taking the maximum expression levels of control (no addition of rare earths) as unity (=1).

Table 2 Genes of *S. coelicolor* 1147 analyzed in this study

Gene[a]	*Product*	*Secondary metabolite–biosynthetic gene cluster*[b]	*Reference*
SCO0124	Hypothetical protein	Eicosapentaenoic acid (type I iterative PKS, SCO0124–0129)	Bentley *et al.*[2]
SCO0381	Putative glycosyl transferase	Unknown (deoxysugar, SCO0381–0401)	
SCO0489	Conserved hypothetical protein	Coelichelin (NRPS, SCO0489–0499)	Lautru *et al.*[19]
SCO1207	Putative cytochrome P450	Tetrahydroxynaphthalene (type III PKS, SCO1206–1208)	Bentley *et al.*[2]
SCO1268	Putative acyltransferase	Unknown (type II fatty acid synthase, SCO1265–1273)	
SCO2785	Conserved hypothetical protein	Desferrioxamines (siderophore synthetase, SCO2782–2785)	Bentley *et al.*[2]
SCO3215	Hypothetical protein	CDA (NRPS, SCO3210–3249)	Bentley *et al.*[2]
SCO5085 (*act*II-ORF4)	Actinorhodin cluster activator protein	Actinorhodin (type II PKS, SCO5071–5092)	Bentley *et al.*[2]
SCO5223	Putative cytochrome P450	Unknown (sesquiterpene synthase, SCO5222–5223)	
SCO5800	Conserved hypothetical protein	Unknown (siderophore synthetase, SCO5799–5801)	
SCO5877 (*redD*)	Transcriptional regulator RedD	Prodiginines (NRPS; type I modular PKS, SCO5877–5898)	Bentley *et al.*[2]
SCO6283	Conserved hypothetical protein	Unknown (type I modular PKS, SCO6273–6288)	
SCO6430	Hypothetical protein	Unknown (NRPS, SCO6429–6438)	
SCO6766	Conserved hypothetical protein	Hopanoids (squalene-Hopene cyclase, SCO6759–6771)	Bentley *et al.*[2]
SCO6826	Conserved hypothetical protein	Unknown (type I modular PKS, SCO6826–6827)	
SCO7670	Conserved hypothetical protein	Unknown (type III PKS, SCO7669–7671)	
SCO7684	Conserved hypothetical protein	Coelibactin (NRPS, SCO7681–7691)	Bentley *et al.*[2]

Abbreviation: CDA, calcium-dependent antibiotic.
[a]Gene names are from Bentley *et al.*[2]
[b]Obtained from Challis and Hopwood.[20]

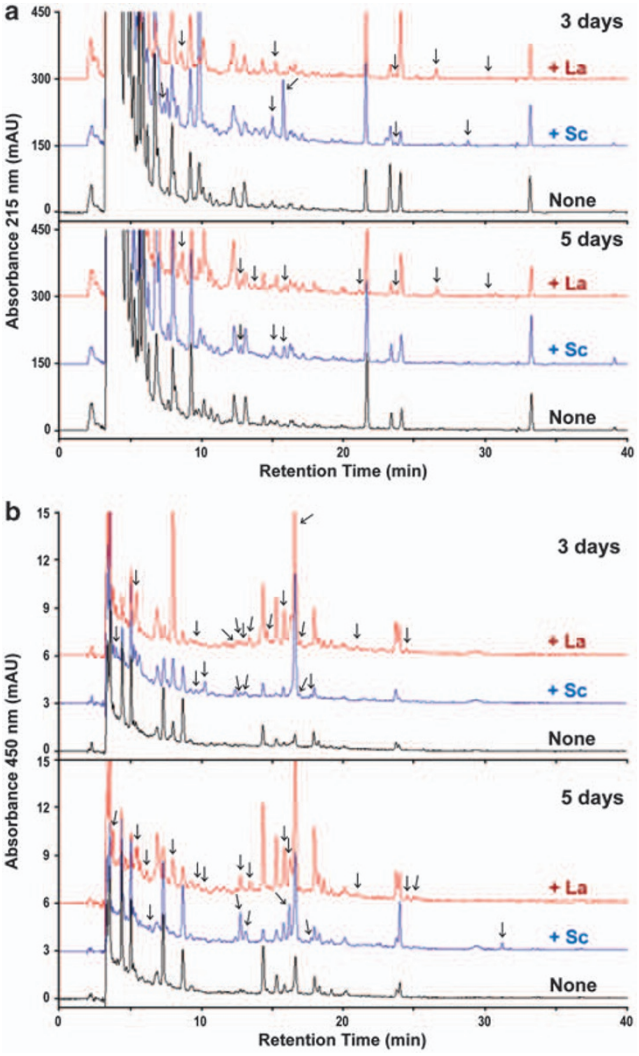

Figure 3 Comparative metabolic profiling of the culture extracts. *S. coelicolor* 1147 was grown on GYM agar medium in the presence or absence of scandium or lanthanum. The culture extracts with EtOAc were prepared after 3 or 5 days of incubation and analyzed by HPLC. The absorbance of the eluate was monitored at 215 nm (**a**) and 450 nm (**b**). The arrows indicate the peaks that were scarcely detected in the control (no addition of rare earths).

SCO1268 (3-fold), SCO2785 (6-fold), SCO3215 (3-fold), SCO5800 (2.5-fold), SCO6283 (4-fold), SCO6826 (3.5-fold) and SCO7684 (2.5-fold). It is notable that, among these genes, SCO1268, SCO5800, SCO6283 and SCO6826 belong to gene clusters, metabolic products of which have not yet been identified (Table 2).

HPLC profile of culture extract

We next compared the metabolic profile of the culture by analyzing the EtOAc-extractable metabolites by HPLC monitoring at different wavelengths (215 and 450 nm). Several peaks (indicated by arrows in Figure 3) were detected only when the strain 1147 was grown in the presence of scandium (200 μM) or lanthanum (1900 μM). Although each peak detected in the presence of rare earths was not identified in this study, the appearance of these peaks reflects the markedly enhanced expression of secondary metabolite–biosynthetic genes (see Figure 2).

CONCLUSION

Antibiotic biosynthesis in streptomycetes is a developmentally regulated process, with production associated with the stationary phase.[11,21] We showed that scandium and lanthanum are both effective not only for enhancement of actinorhodin production but also for activation of silent or poorly expressed genes in *S. coelicolor*. In particular, scandium appears to be a feasible reagent for gene activation because it showed a rather wide range of effective concentrations. Another advantage of using rare earths is that this method does not require any gene engineering technology or genomic information for the strains examined. As rare earths are distributed ubiquitously throughout the world, it is conceivable that microorganisms have acquired the ability to respond to low levels of these elements over the course of their long evolutionary history, possibly as a means of adapting their physiology to the prevailing conditions. The compelling effect of low levels of scandium on antibiotic production implies that scandium functions *in situ* as a factor that induces or stimulates the production of secondary metabolites, which can include pigments, mycotoxins, phytotoxins and antibiotics. The mechanism of action, however, remains to be clarified. The present method, together with other methods reported recently,[8,9,12,22–24] may be useful for activating silent genes, eventually leading to the discovery of novel biologically active compounds.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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