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ORIGINAL ARTICLE

Divergent effects of desferrioxamine on bacterial growth and characteristics

Daisei Eto, Kenta Watanabe, Hisafumi Saeki, Ken-ichi Oinuma, Ko-ichi Otani, Megumi Nobukuni, Hatsumi Shiratori-Takano, Hideaki Takano, Teruhiko Beppu and Kenji Ueda

Desferrioxamines (DF's) are siderophores produced by some groups of bacteria. Previously, we discovered that DFE, produced by *Streptomyces griseus,* induced divergent developmental phenotypes in various *Streptomyces* isolates. In this study, we isolated bacteria whose phenotype was affected by the presence of 0.1 mM DFB from soil samples, and studied their phylogenetic position via 16 S rRNA gene-based analysis. Isolates belonging to *Microbacterium* grew only in the presence of DFB in the medium. DFB promoted growth of some isolates, while significantly inhibiting that of other divergent bacteria. Different groups of isolates were affected, not because of growth-related changes, but because of changes in the colony morphology based on possible stimulation of motility. An isolate affiliated with *Janthinobacterium* was stimulated for violacein production as well as for pilus formation. The wide and divergent effects of DFB suggest that availability of siderophores significantly affect the structure of microbial community.
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INTRODUCTION

Interactions among microorganisms constitute a fundamental characteristic of the ecosystem. These interactions include various kinds of relationships that promote and inhibit the proliferation and/or expression of certain microbial functions. A detailed characterization of such interactions is expected to provide significant insights into the foundation of microbial ecology and biotechnology; however, these barely visible interactions have not been effectively observed.

Previously, we identified desferrioxamine (DF), a siderophore widely produced by *Streptomyces*, to be responsible for increased growth and/or effective development in some *Streptomyces* spp.[1] Originally, our extensive screening for interaction between different *Streptomyces* spp. showed that antibiotic production and/or morphological differentiation in a wide variety of strains was stimulated by cocultivation with different strain. Of these, we observed that cocultivation of *Streptomyces griseus* with *Streptomyces tanashiensis* caused marked growth of the latter due to a diffusible metabolite produced by the former. Our study revealed that the growth-promoting substance produced by *S. griseus* was DFE.[2] Furthermore, we discovered that exogenous supply of this type of siderophore affected various characteristics of *Streptomyces* spp., including growth promotion, cell differentiation and/or secondary metabolite formation. This suggested that DF's are widely utilized by this group of bacteria to activate specific cellular functions.

It is generally understood that siderophores are involved in the acquisition of ferric ions.[3] Despite its wide occurrence in the natural environment, the insoluble nature of iron is responsible for its limited availability to cells. Microorganisms acquire ferric iron by secreting a siderophore, and subsequently uptaking it as a complex with ferric iron. To date, various siderophores and membrane translocators involved in the uptake of those siderophores have been identified.[3] On the other hand, the above-mentioned dependence of *S. tanashiensis* on exogenous DF for growth implies that some microbial populations lack the ability to synthesize siderophores, but retain the mechanism to utilize the siderophores produced by other microbes.[2]

In this study, we screened for bacterial strains whose growth characteristics were affected by the exogenous supply of DFB, the medically utilized siderophore produced by *Streptomyces pilosus.*[4] The group of siderophore has long been known as a growth factor for some specific bacterial strains such as *Arthrobacter* spp.,[5] but its diverged effect on microbial physiology has not yet been fully studied. The fact that the uptake of the siderophore takes place even in the organisms that do not have the ability to synthesize it[2] makes us think of the possibility that the siderophore produced by *Streptomyces* significantly affects the biological activities in the natural environment. Here, we found that DFB had various effects not only on growth but also on specific functions in taxonomically divergent bacteria. This evidence implies that this type of siderophore serves as an association factor that widely affects microbial population dynamics and functional expression.

Life Science Research Center, College of Bioresource Sciences, Nihon University, Kanagawa, Japan
Correspondence: Dr K Ueda, Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan.
E-mail: ueda.kenji@nihon-u.ac.jp
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MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

DFB-dependent bacteria were isolated from soil samples collected at Fujisawa City (Kanagawa, Japan). Appropriately diluted soil suspensions were first inoculated onto 1/100 Luria–Bertani (LB) medium containing 0.01% Bacto Tryptone (Difco, Detroit, MI, USA), 0.005% Bacto Yeast extract (Difco), 0.005% NaCl, 10 μM $FeCl_3$ (pH was adjusted to 7.2 by NaOH), 0.1 mM DFB (Sigma-Aldrich, Tokyo, Japan; if required) and 1% agar (chemicals were purchased from Kokusan, Tokyo, Japan, if not indicated otherwise) and cultured for 3–10 days at 28 °C. The use of diluted LB medium enabled isolation of diverged bacteria, as it reduced the growth rate of rapid growers. Resultant bacterial colonies were then plated in duplicate onto 1/100 LB medium or LB agar medium containing 1% Bacto Tryptone, 0.5% Bacto Yeast extract, 0.5% NaCl, 10 μM $FeCl_3$ (pH 7.2) and 1% agar, supplemented with or without 0.1 mM DFB, and cultured for 3–10 days at 28 °C. Isolates whose growth or some other property was affected by the presence of DFB were selected, and the dependence was confirmed by repeated cultivation. Cells were inoculated by spreading and streaking on the agar medium, and the condition effectively showing the dependence was used. To determine the minimum DFB concentration for growth promotion, KW016 cells were precultured in LB liquid medium containing 1 μM DFB by shaking reciprocally at 300 r.p.m. at 28 °C for 3 days, washed twice with LB medium, and inoculated at 1% (vol/vol) into fresh LB liquid medium supplied with various concentrations of DFB. To assess for IC50 values, the strains of complete inhibition group were precultured in LB liquid medium by shaking reciprocally at 300 r.p.m. at 28 °C for 3 days and inoculated at 1% (vol/vol) into fresh LB liquid medium supplied with various concentrations of DFB. Growth was studied by measuring OD ($\lambda = 600$).

The following bacterial strains were obtained from National Bioresource Center, NITE, Japan: *Chromobacterium violaceum* NBRC 12614, *Janthinobacterium lividum* NBRC 12613, *Collimonas* spp. NBRC 3740, *Microbacterium flavescens* NBRC 15039, *Microbacterium testaceum* NBRC12675, *Microbacterium trichothecenolyticum* NBRC15077, *Microbacterium foliorum* NBRC12124, *Microbacterium oxydans* NBRC15586, *Microbacterium barkeri* NBRC15036, *Microbacterium arabinogalactanolyticum* NBRC14344, *Microbacterium chocolatum* NBRC3758 and *Microbacterium imperiale* NBRC12610. *Escherichia coli* JM109 and pUC19 (Takara-shuzo, Kyoto, Japan) were used as the host and vector for general DNA manipulation. Conditions for the culture of *E. coli* were previously described.[6]

Taxonomic characterization

To study the 16 S rRNA gene sequence of the isolate, DNA was extracted using a bacterial genomic DNA purification kit (Edge Biosystems, MD, USA). Nearly complete 16 S rRNA gene fragments were amplified by PCR using primers B8F (5′-AGAGTTTGATCCTGGCTCAG; nt 8–27 based on *Escherichia coli* numbering) and B1492R (5′-GGTTACCTTGTTACGACTT; nt 1508–1484 based on *E. coli* numbering). PCR was performed on a T1 Thermocycler (Biometra, Gottingen, Germany), with Ex *Taq* polymerase (Takara-shuzo). The PCR protocol included an initial denaturation period of 4 min at 94 °C; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; 3 min at 72 °C; and incubation at 4 °C until further processing. The 16 S rRNA gene fragment was directly sequenced by using the BigDye terminator v3.1 cycle sequencing kit on an ABI 3130 genetic analyzer (Applied Biosystems Japan, Tokyo, Japan). The 16 S rRNA sequences were compared with those from the GeneBank/EMBL/DDBJ nucleotide sequence database, by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Scanning electron microscopy

The DA027 strain, cultured at 28 °C for 3 days on LB medium, was observed using scanning electron microscopy. To prepare specimens, agar blocks were fixed with 2% osmium tetroxide for 30 h, and then dehydrated by freeze-drying. Each specimen was sputter coated with palladium/gold by using an E-1010 ion sputter (Hitachi, Tokyo, Japan) and scanned under a VE8800 scanning electron microscope (Keyence, Tokyo, Japan).

Pigment extraction

The DA027 cells, grown at 28 °C for 3 days on LB agar medium containing 0.1 mM DFB, were collected from the medium surface and used to isolate the purple pigment. The pigment was extracted by treating the cells with diethylether. After concentration by evaporation, the extract was dissolved in appropriate amount of ethanol and applied onto a thin layer chromatography plate (C18; Merck Japan, Tokyo). Violacein similarly extracted from the cells of *C. violaceum* NBRC 12614 grown at 28 °C for 3 days on LB agar medium was used as a standard. The thin layer chromatography plate was developed with ethanol:water = 6:4.

RESULTS

Approximately 8000 bacterial strains isolated from soil samples using diluted LB medium were studied for their dependence on the exogenous supply of DFB (Materials and methods). The divergent effects of DFB included both promotion and inhibition of growth, as well as alteration of specific properties such as colony morphology and pigment formation (appearances of the representative colonies of DFB-dependent isolates are shown in Figure 1). The DFB-dependent bacteria were assessed for their taxonomic affiliation by 16 S rRNA gene-based phylogenetic analysis. The results are summarized in Table 1.

All three isolates that were completely dependent on DFB for growth belonged to *Microbacterium* (Table 1, a representative strain KW016 is shown in Figure 1). On the basis of this finding, nine species of *Microbacterium* were obtained from NBRC culture collection (see materials and methods), and studied for their dependence on DFB. The results show that all bacteria, except *M. flavescens*, effectively grew on medium without DFB (data not shown). *M. flavescens* NBRC 15039 exhibited marked dependence on DFB, similar to what the three isolates obtained in this study exhibited

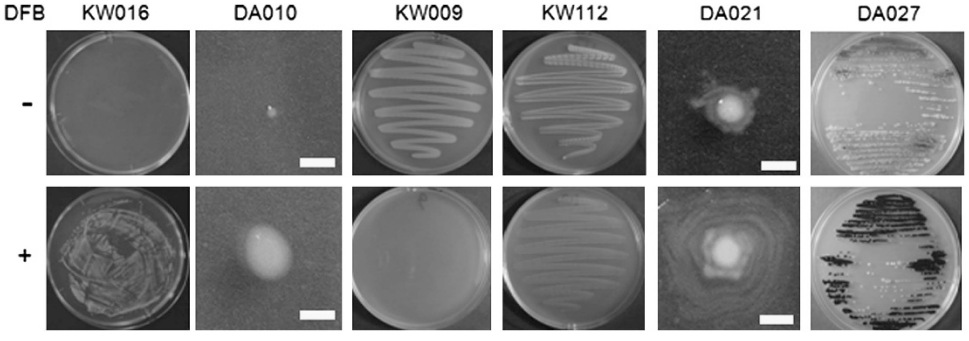

Figure 1 Typical DFB-dependent bacterial phenotypes observed in this study. Representative colony of strains were developed by cultivating them for 3 days at 28 °C on LB medium containing 10 μM $FeCl_3$ without (−) and with (+) the supply of 0.1 mM DFB. Bar, 5 mm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 DFB-dependent isolates obtained in this study

		Closest identified relative		
Strain Name [a]	*Read(nt)*	*Taxon*	*Identity (%)*	*Accession no.*
Isolates whose growth essentially required DFB				
KW016	1369	*Microbacterium phyllosphaerae*	98.8	EU714359
KW111	1381	*Microbacterium flavescens*	98.8	Y17232
KW080	1383	*Microbacterium flavescens*	98.8	EU714363
Isolates whose growth was promoted by DFB				
KW040	1402	*Burkholderia cepacia*	99.9	GQ383907
KW096	1369	*Gordonia alkanivorans*	99.7	AY995556
KW105	1388	*Burkholderia fungorum*	98.6	AB091188
DA010	1510	*Paenibacillus nanensi*	94.0	AB265206
Isolates whose growth was completely inhibited by DFB				
KW009	1315	*Shewanella putrefaciens*	99.3	GQ359955
KW018	1363	*Bacillus shandongensis*	99.9	EU046267
KW019	1405	*Sporosarcina aquimarina*	98.1	EU308120
KW031	561	*Dermacoccus nishinomiyaensis*	100	AJ627008
KW034	1400	*Staphylococcus epidermidis*	100	FJ768459
KW046	1351	*Bosea thiooxidans*	99.5	AF508803
KW047	1309	*Nitrobacteria novellus*	99.2	DQ205093
KW049	1386	*Arthrobacter humicola*	99.9	AB279890
KW051	501	*Arthrobacter globiformis*	99.5	EF634027
KW055	1408	*Staphylococcus epidermidis*	99.9	FJ357583
KW060	1257	*Pseudoxanthomonas mexicana*	99.5	EU294417
KW065	896	*Phenylobacterium immobile*	97.5	Y18216
KW066	1350	*Sphingopyxis panaciterrae*	97.9	EU730911
Isolates whose growth was partially inhibited by DFB				
KW032	989	*Lysobacter niastensis*	98.5	DQ462462
KW112	670	*Mycobacterium lacticola*	99.5	AF480582
Isolates whose pigment production was promoted by DFB				
KW001	1353	*Pseudomonas putida*	99.9	AY918067
KW003	1309	*Pseudomonas fluorescens*	99.9	DQ095891
KW122	1377	*Streptomyces californicus*	99.8	AY999845
DA027	1495	*Janthinobacterium lividum*	99.5	EU275366
Isolates whose colony morphology was affected by DFB				
KW103	1392	*Burkholderia ambifaria*	99.5	CP001025
KW128	1384	*Streptomyces lipmanii*	99.5	AB045861
KW133	1366	*Mycobacterium frederiksbergense*	99.5	AJ276274
DA003	1300	*Lysinibacillus sphaericus*	99.5	AB116123
DA018	1506	*Paenibacillus macerans*	96.2	AB363744
DA021	1489	*Paenibacillus ginsengagri*	98.2	AB245383

[a]Nucleotide sequences exhibiting less than 99% identity with known sequences were deposited to DDBJ database under following accession numbers: KW016, AB686541; KW111, AB686542; KW080, AB686543; KW105, AB686544; DA010, AB489860; KW019, AB686545; KW066, AB686546; DA018, AB489861; DA021, AB489862.

(data not shown). The minimal concentration of DFB for the growth promotion in KW016 strain was determined to be ca. 0.1 μM.

Other four diverged bacterial isolates affiliating with *Burkholderia*, *Gordonia* and *Paenibacillus* (*Paenibacillus* DA010 is shown in Figure 1), grew on the medium without DFB, but their growth was significantly promoted by the presence of DFB. In contrast, the supply of DFB completely or partially inhibited the growth of many divergent bacteria; (for example, KW009 and KW112, Figure 1 and Table 1) including both gram-negative and –positive bacteria. We studied the IC50 values with respect to the strains of complete inhibition group, and found that the values were between 50 and 70 μM.

The effects of DFB included not only growth promotion/inhibition but also alteration of colony morphology and induction of pigment production (Table 1). DA021 strain closely related to *Paenibacillus* formed a diffusible colony in the DFB-containing medium, probably because of promotion of motility (Figure 1). Similar stimulation of motility was observed with respect to KW133, DA003 and DA018 (Supplementary figure S1). The colony of KW103 exhibited a smooth surface on DFB-plus medium although it showed rough surface on DFB-minus one. KW128, a *Streptomyces* strain, abundantly formed aerial mycelium and spores in DFB-plus condition as observed previously with regard to some strains of the same bacterial group[2] (Supplementary figure S1).

DA027 strain belonging to *J. lividum* effectively produced purple pigment in the DFB-containing medium (Figure 1). DFB promoted production of a yellow intracellular pigment in strains KW001 and 003, and a purple pigment in KW122 strain (Supplementary figure S1).

Figure 2 shows the stimulation of production of purple pigment in DA027 and related strains obtained from the culture collection center. Similar promotion of pigment production was observed in *J. lividum* NBRC12613 and *Collimonas* spp. NBRC3740 (Figure 2a). Scanning electron microscopy revealed that the DA027 cells grown in the presence of DFB effectively formed pili (Figure 2b). Thin layer chromatography analysis of the purple pigment extracted from the cells of DA027 strain identified it to be violacein, a pigmented antibiotic produced by some gram-negative bacteria including *C. violaceum* (data not shown).

DISCUSSION

Many microorganisms cope with extreme iron limitations in the natural environment by synthesizing siderophores.[3] The producer organisms secrete the siderophore(s) and uptake the substance(s) in ferric-bound form, via specific transport mechanisms. These microbes carry out this energy-wasting process because of the necessity of acquiring ferric iron for cellular ferric-dependent functions.

This study showed that siderophore production is not just a self-supporting process in a specific microorganism, but an event that affects the physiology of other microorganisms too. Although the wide distribution of siderophore transporter genes suggests that uptake of the siderophores produced by other bacteria generally occurs in bacteria,[7] the actual effects have not yet been extensively surveyed. The divergent effects caused by DFB, including stimulation and inhibition of growth and induction of specific functions, suggest that the availability of siderophores significantly affects the dynamics of the microbial community in nature.

The isolates belonging to *Microbacterium* strictly depend on the supply of DFB for growth. *M. flavescens* (formerly described as *Arthrobacter flavescens*), with which the isolates obtained in this study (KW111 and KW080; Table 1) were closely related, has been known for its strict growth dependence on a supply of ferrichrome or nocardamine (synonym of desferrioxamine).[8] The DFB-independent growth of many other species of *Microbacterium* indicates that the requirement is a specific feature of the limited taxonomic group of the

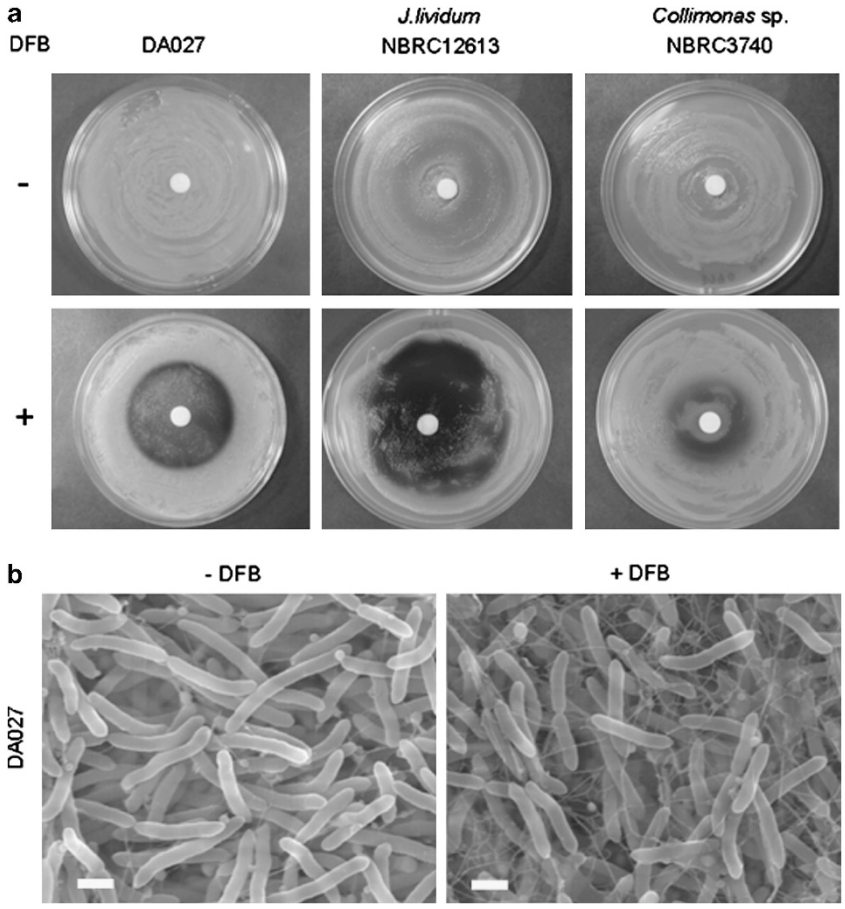

Figure 2 DFB-induced violacein production (**a**) and pilus formation (**b**) in DA027. (**a**) Filter discs containing 0 (−) and 2.8 mg (+) of DFB were placed into the LB agar inoculated with DA027 and related bacterial strains, and the bacteria were incubated for 3 days at 28 °C. (**b**) Scanning electron micrographs of the DA027 strain grown under the same conditions as in (**a**). The photograph of the DFB(+) condition shows the appearance of the violacein-producing cells. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

genus. Probably, these bacteria lack the ability to synthesize siderophores, but they are capable of uptaking and utilizing ferrioxamines. This suggests that the bacteria syntrophically grow by associating with the microbial community producing DFB and related siderophores in the natural environment.

This strict dependence observed with respect to the *Microbacterium* spp. indicates that some siderophores should be added to culture medium in order to isolate a particular type of bacteria. Recently, D'Onofrio *et al*.[9] reported that supply of the siderophore acyl-DF enabled cultivation of previously uncultured isolates. This as well as our current evidence strongly reinforces the view that the conditions of ferric supply are an important factor with respect to microbial screening.

In contrast to the positive effects, DFB inhibited the growth of a wide variety of bacteria. Presumably, this inhibition is due to the sequestration of ferric iron by DFB. It is supported by the relatively high level of IC50 values (50–70 μM). Since the dose is probably higher than the actual content of this kind of siderophore in the natural environment, the growth inhibition observed in our studies may not be a naturally occurring phenomenon. However, available evidence supports the view that these isolates do not contain the uptake system for DFB, and that they obtain ferric iron on the basis of the function of another type(s) of siderophore and/or a different uptake mechanism. This further makes us speculate that the microbial population in the environment consists of multiple siderophore-utilizing groups, and that the commensalism within each group as well as the competition between different groups serves as a basis for microbial population dynamics in terms of ferric iron utilization.

The addition of DFB caused divergent effects not only on growth but also on specific phenotypes of the bacterial isolates. The colony morphology of some isolates was affected by DFB, possibly due to the stimulation of motility. In *Vibrio cholerae*, a ferric-depleted condition induces the transcription of genes, including those involved in motility and chemotaxis, on the basis of the function of the ferric-dependent repressor Fur and small regulatory RNA RyhB.[10] Although the mechanism of DFB-dependent motility observed in this study is not yet clear, it is possible that depletion of ferric iron due to the sequestration by DFB induces the tactic behavior in some bacteria. Another possibility is that the availability of ferric-DFB complex serves as a positive factor inducing motility in some bacteria. Future characterization is required to understand the mechanism of DFB-dependent motility.

Notable stimulation was observed with respect to violacein production in *Janthinobacterium* and related gram-negative bacteria. Our preliminary study on the expression of violacein biosynthesis (*vio*) genes, indicated that the addition of DFB does not affect the transcription level of *vio* genes in *J. lividum*, indicating that the supply of DFB affects some post-transcriptional step for violacein synthesis (our unpublished observation). One of the biosynthetic enzymes, VioB, which catalyzes tryptophan dimerization, is a heme-containing enzyme.[11] Acquisition and supply of ferric iron to this biosynthetic

enzyme based on the availability of DFB could be the reason for DFB-dependent production of violacein.

We discovered that the supply of DFB-induced marked pilus formation in DA027 strain (Figure 2b). To date, the physiological role of pilus formation in this gram-negative bacterium is not known. However, we can reasonably speculate that frequent pilus formation facilitates cell-to-cell and cell-to-surface associations in this organism. The availability of abundant ferric iron due to the presence of DFB may serve as a positive signal for colony development based on the cellular interactions in some bacteria. Future detailed analyses with respect to the effects of DFB and other siderophores will provide useful information regarding the social behavior of microorganisms.

CONFLICT OF INTEREST

The authors declare no conflict of interst.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)