A key developmental regulator controls the synthesis of the antibiotic erythromycin in *Saccharopolyspora erythraea*

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*Saccharopolyspora erythraea* makes erythromycin, an antibiotic commonly used in human medicine. Unusually, the erythromycin biosynthetic (ery) cluster lacks a pathway-specific regulatory gene. We isolated a transcriptional regulator of the ery biosynthetic genes from *S. erythraea* and found that this protein appears to directly link morphological changes caused by impending starvation to the synthesis of a molecule that kills other bacteria, i.e., erythromycin. DNA binding assays, liquid and affinity chromatography, MALDI-MS analysis, and de novo sequencing identified this protein (*M* = 18 kDa) as the *S. erythraea* ortholog of BldD, a key regulator of development in *Streptomyces coelicolor*. Recombinant *S. erythraea* BldD bound to all five regions containing promoters in the ery cluster as well as to its own promoter, the latter with an order-of-magnitude stronger than to the ery promoters. Deletion of *bldD* in *S. erythraea* decreased the erythromycin titer in a liquid culture 7-fold and blocked differentiation on a solid medium. Moreover, an industrial strain of *S. erythraea* with a higher titer of erythromycin expressed more BldD than a wild-type strain during erythromycin synthesis. Together, these results suggest that BldD concurrently regulates the synthesis of erythromycin and morphological differentiation. The ery genes are the first direct targets of a BldD ortholog to be identified that are positively regulated.

Erythromycin, an antibiotic made by *Saccharopolyspora erythraea*, kills Gram-positive bacteria that infect humans. The industrial importance of erythromycin is significant, with worldwide sales of erythromycin and its derivatives reaching billions of dollars every year. Two strategies, classical strain improvement and rational engineering, have boosted titers of erythromycin from *S. erythraea*. In classical strain improvement, a strain undergoes multiple rounds of random mutagenesis and screening. Although tedious and time-consuming, the strategy can increase titers 10- to 100-fold (1). In rational engineering, knowledge of biochemical pathways guides genetic manipulations to increase titers, such as through modification of the methylmalonyl-CoA metabolite node (2). Often, overexpression of a gene that regulates the biosynthetic cluster of an antibiotic increases titers. For instance, overexpression of *sanG*, a transcriptional regulator of the nikkomycin biosynthetic genes in *Streptomyces ansochromogenes*, increased titers of the antibiotic (3). Normally, such regulatory genes, like actII-ORF4 for actinorhodin (4) or redD for undecylprodigiosin (5) in *Streptomyces coelicolor*, lie within the antibiotic biosynthetic cluster. However, in *S. erythraea*, the biosynthetic cluster for erythromycin lacks a regulatory gene.

In actinomycetes, synthesis of antibiotics often coincides with complex morphological changes during the life cycle. Compounds produced during these stages of differentiation comprise nearly two-thirds of bioactive molecules synthesized by microorganisms, including antibiotics, antitumor agents, and immunosuppressants. *S. coelicolor*, among the most well studied of actinomycetes, produces several antibiotics and has a sequenced genome (6). Apart from the pathway-specific regulators for antibiotic biosynthesis, other genes of *S. coelicolor* have pleiotropic roles in development and the synthesis of antibiotics. For example, mutation or deletion of *bldD* causes defects both in the formation of aerial hyphae and the production of antibiotics (7). To our knowledge, none of the genes with characterized roles in the development of *S. coelicolor* directly regulate an antibiotic biosynthetic gene cluster.

Previously, DNA microarrays revealed differences in transcription between two *S. erythraea* strains, a wild-type (WT) strain and a classically improved strain that overproduces erythromycin (OVP). In particular, the OVP strain expressed the entire biosynthetic cluster of erythromycin (ery) several days longer than the WT strain (8). The altered and coordinated expression of nearly all of the *ery* genes suggested the existence of a common regulator for the *ery* cluster, although the genome sequence of *S. erythraea* confirmed the absence of such a regulator within the cluster (9). In this study, we purified and identified a regulatory protein, BldD, which binds to all of the promoters in the *ery* cluster. This work reveals multiple cellular roles of BldD and makes progress toward the rational manipulation of actinomycetes to overproduce antibiotics.

Results and Discussion

**Protein Binding to the Promoters of the Erythromycin Biosynthetic Gene Cluster.** Sustained, coordinate expression of the biosynthetic gene cluster of erythromycin by the OVP strain indicated the existence of a global regulator of these genes (8). To begin to identify the protein, we prepared fluorescent DNA probes of the five regions containing ery promoters (Fig. L4) (10, 11). When incubated with crude lysates of both WT and OVP strains after 22 and 65 h of growth in liquid medium, all five promoter probes showed shifts in electrophoretic mobility shift assays (EMSA), with *eryIV-BIV* and *eryBVI* probes producing the strongest shifts with the 22 h lysates (Fig. 1B, lanes c and e). For both WT and OVP strains, there was no shift observed after 65 h of growth (Fig. 1B, lanes d and f). The *eryK* and *eryCl ermE* probes exhibited only modest shifts, whereas a smeared shift was observed for the *eryB1-BIII* probe with all lysates tested [supporting information (SI) Fig. S1]. Moreover, we obtained similar EMSA results by using DNA probes prepared from genomic DNA of both WT and OVP strains (data not shown).

Several tests indicated that the protein(s) causing the shifts bound to *ery* promoters specifically. In competition with the
Fig. 1. EMSAs with promoters of the ery cluster and lysates of S. erythraea. (A) The biosynthetic gene cluster of erythromycin (ery). Gray lines show regions that contain promoters and were used as probes for EMSAs. For eryBVI and eryK, probes included the start site of transcription and the start codon. For divergent promoters (eryCl-ermE, eryB-BlI, and eryAI-BIV), probes included start sites of transcription and start codons of the divergent genes. (B) EMSAs for promoters eryAI-BIV and eryBVI. Each probe has lanes b–f. Lanes show DNA ladder (a), probe only (b), probe + WT 22-hr lysate (c), probe + WT 65-hr lysate (d), probe + overproducer 22-hr lysate (e), and probe + overproducer 65-hr lysate (f). (C) EMSAs with the eryBVI probe and lysates of the WT and OVP strains at 12, 24, 30, and 48 h. Results are a representative example of three independent experiments.

erBVI probe, excess unrelated DNA (a fragment of a plasmid that contains an oriT site) did not affect the shift of the probe, but excess unlabeled eryBVI probe effectively competed with the labeled probe for binding to BldD (data not shown). In addition, incubation of a crude lysate of S. erythraea with a promoter from S. coelicolor (actVI-Ap) produced no shift (data not shown). Together, these initial results suggested that the protein(s) causing the EMSA shifts bind specifically to promoters of the ery cluster.

Presence of BldD Correlates with Expression of the ery Cluster. A time course revealed more precisely when the WT and OVP strains produce the protein(s) causing the EMSA shifts in vegetative cultures. The two strains had similar growth curves in a liquid, rich medium (Fig. S2). In EMSAs with the eryBVI probe, lysates from both strains produced a shift at 12 h, the earliest time point (Fig. 1C). For the WT strain, the shift was observed until 24 h. In contrast, for the OVP strain, the shift remained detectable at 30 h (Fig. 1C), roughly correlating with the lengthened expression of the ery cluster.

Regulator Is a BldD Ortholog. A protein designated BldD (see above) was purified based on its ability to cause an EMSA shift by using the eryBVI probe, and its structural gene was cloned as described in Materials and Methods. The bldD gene encodes a protein of 162 aa and molecular mass of 17.7 kDa (Fig. S3). The predicted BldD sequence yielded strong matches to the BldD molecule megalomicin, suggests that the BldD of Streptomyces coelicolor. This finding was unexpected because, although mutations in S. coelicolor bldD influence the synthesis of secondary metabolites (12), we found no published evidence indicating that a member of the BldD family directly regulates antibiotic biosynthetic genes. In S. coelicolor, BldD negatively regulates expression of the developmental σ factor genes bldN and whiG during vegetative growth (13), in addition to repressing vegetative expression of the stress response σ factor gene sigH (14) and an as-yet uncharacterized regulator termed bdtA (13). It also negatively regulates its own synthesis (15). Thus, all of the BldD targets so far identified in S. coelicolor are repressed by BldD, whereas the ery genes are positively regulated by BldD in S. erythraea.

BldD Resides Apart from the ery Cluster in the Circular Chromosome of S. erythraea. In the 8.2-Mb S. erythraea chromosome, the ery cluster is centrally located within the core region, which encodes most of the essential genes (9), whereas bldD lies near the edge of the core region, ~1.5 Mb from the ery cluster (Fig. 2A). The separate positions of bldD and the ery cluster in the chromosome of S. erythraea contrast with many biosynthetic clusters of antibiotics such as actinorhodin (4) and undecylprodigiosin (5) that contain regulatory genes. Analysis of sequences for Aeromicrobium erythreum, which also synthesizes erythromycin, and Micromonospora megalomica, which synthesizes the related molecule megalomicin, suggests that the ery cluster once might have contained a regulatory gene. Both species have biosynthetic gene clusters extremely similar to the ery cluster in S. erythraea

Fig. 2. Chromosomal locus of bldD. (A) Location of bldD in the chromosome of S. erythraea. Gray bar indicates the core region. (B) Alignment of the locus of bldD (S. ery) to the locus of bldD in S. coelicolor (S. coe). Black arrows show bldD (S. ery) and bldD (S. coe). Alignments represent genes homologous between the two bacteria. The dotted line represents a discontinuity of one gene in S. coelicolor in the alignment. The numbers above the genes are gene numbers in the genome sequence for both species.
but contain putative regulatory genes. In *A. erythreum*, a transcriptional regulator of the MarR family (ery-ORF25) is encoded at one end of the *ery* cluster adjacent to *eryCI* (16), and in *M. megalomica*, a putative regulator is encoded at the end end adjacent to megDVI (17).

The gene organization around the *bldD* gene is very similar to that of *bldD* in *S. coelicolor* (Fig. 2B). In particular, the three genes downstream of *bldD* (SACE2074–2076) resemble three genes downstream of *s. coelicolor bldD* (SCO1492–1490), and the six genes upstream of *bldD* (SACE2078–2084) resemble six genes upstream of *S. coelicolor bldD* (SCO1488–1483 and SCO1481). Combined with the bldD phenotype of the *bldD* mutant (see above), this synteny reinforces the idea that *S. erythraea* BldD and *S. coelicolor* BldD are orthologous.

**Recombinant BldD Binds to ery Promoters and to Its Own Promoter.** To confirm that *bldD* encodes a DNA binding protein, we expressed recombinant BldD in *Escherichia coli* BL21(DE3). In EMSAs, a lysate of *E. coli* induced to express BldD shifted the *eryBVI* probe completely, whereas a lysate of an uninduced strain caused no shift (data not shown). Further, a 56-bp footprint of BldD binding at the *eryBVI* region was obtained (Fig. 3 and Fig. S4). The footprint includes the transcriptional start site of *eryBVI* (11), and although it is an unusual binding site for an activator, it has been observed before (18). By visual inspection of the sequence within this protected region, we identified a possible binding sequence of AGTGC(n)9TCGAC for BldD, based on the *S. coelicolor* BldD consensus binding sequence of AGTgA(n)mTCACc (13). Consistent with our data, *S. coelicolor* BldD binds upstream or across the transcriptional start sites of its targets (13–15). However, *S. coelicolor* BldD acts as a transcriptional repressor of each of these target genes (12–14), whereas *S. erythraea* BldD appears to be acting as a transcriptional activator of the *ery* gene cluster. The mechanism by which BldD can act as a transcriptional activator in some cases and a transcriptional repressor in others is unclear and warrants further investigation.

BldD shifted all five probes of the *ery* promoters (Fig. 4), indicating that BldD regulates the entire biosynthetic cluster of erythromycin. To assess the affinity of BldD for each probe, we purified recombinant BldD by using a Ni-NTA resin (data not shown). Titrations of BldD with a fixed concentration of each probe, followed by measurements of the fraction of probe bound in EMSAs, yielded equilibrium dissociation constants (*Kd*) (Table 1 and Fig. S5). BldD binds with similar affinities to the probes of *eryAI-VI*, *eryBVI*, and *eryBI-III*, and ~3- to 5-fold less strongly to the probes of *eryK* and *eryCI-ermE*.

Because *S. coelicolor* BldD binds to its own promoter (15), we asked whether *S. erythraea* BldD bound to a sequence upstream of *bldD*. EMSAs with the *S. erythraea bldD* promoter and purified recombinant BldD resulted in two shifted fragments (Fig. 5A). Both shifts required the presence of BldD, but we were unable to determine whether they arose from different conformations or multimers of BldD. Note that *S. coelicolor* BldD binds to its own promoter as a dimer, also resulting in two distinct bands in EMSA experiments (7). In addition, *Kd* of 0.32 μM for BldD binding to its own promoter was determined, which approximates the *Kd* of BldD for its own promoter (19). Therefore, BldD binds to the *bldD* promoter an order of magnitude more strongly than it does to promoters of the *ery* cluster.

**S. erythraea Overproducer Strain Has More BldD.** Because BldD binds to its own promoter with submicromolar *Kd*, we used the *bldD* promoter to examine how BldD binding varied with a time course of lysates from *S. erythraea*. Cultures of the WT and OVP strains in a liquid, rich medium were sampled at 27, 40, 62, 87, and 111 h. EMSAs using lysates from both strains revealed two shifted probe fragments at 27 h, the initial time point (Fig. 5B). By 40 h, the WT strain lysate failed to produce most of the upper band, whereas the OVP strain lysate still revealed that band at the last time point tested (111 h). Also, the reactions with WT-strain lysates resulted in more unbound probe than with the OVP strain lysates at all times (Fig. 5B). To determine whether the greater shifts observed with OVP strain lysates were because

![Fig. 4. EMSAs with recombinant BldD and the five regions of *ery* promoters, designated *eryCI-ermE*, *eryAI-VI*, *eryAI-VI*, and *eryK*. +, probe only; +, probe and BldD.](https://www.pnas.org/cgi/doi/10.1073/pnas.0803622105 Chng et al.)
of more abundant BldD, we assessed the expression of BldD in the WT and OVP strains by using Western blots. Polyclonal anti-BldD antibody detected BldD in the first four lysates of each previous time course. BldD was observed in both strains after 27 h of growth, when they were producing erythromycin (Fig. 5 C and D). However, BldD abundance in the WT strain decreased after 40 h, coinciding with a decrease in erythromycin production. In contrast, the OVP strain maintained relatively constant BldD levels up to 87 h, as production of erythromycin continued. The amounts of BldD detected by Western blot analysis matched well with the intensities of shifts in EMSAs (Fig. 5 B), indicating that a higher abundance of BldD, rather than a more active form, caused stronger shifts from the OVP strain. How the OVP strain acquires the phenotype of extended BldD expression is not known, but we postulate that during classical strain improvement, mutations were introduced in genes that regulate BldD expression, as the sequences for the promoter and coding region of bldD are identical for the WT and OVP strains.

**Deletion of bldD Generates a ”Bald” Phenotype.** We deleted bldD in S. erythraea strain AML315–638 (which derives from the WT strain) (20) as described in Materials and Methods. The deletion strain (ΔbldD) failed to form aerial mycelium and to sporulate on three different media, M1 (21), SFM, and R5 agar (Fig. 6 A and Fig. S6). This bald phenotype, which is also characteristic of S. coelicolor ΔbldD strains (7, 22), suggests that S. erythraea BldD and S. coelicolor BldD have similar functions. Complementation of the bldD deletion with a single copy of bldD restored the WT phenotype, whereas complementation with a plasmid lacking bldD maintained the bald phenotype (Fig. 6 A). When grown in a liquid, rich medium for 5 days, the ΔbldD strain produced 7-fold less erythromycin than the WT strain (Fig. 6 B). Complementation of the bldD deletion with a single copy of bldD restored normal titers, whereas complementation with a plasmid lacking bldD left titers low (data not shown). Together, these data suggest that BldD positively regulates the ery genes.

However, because the mutation left some synthesis of erythromycin intact, it differs from deletions of activators in many streptomycetes that abolish the production of an antibiotic completely (3, 23). Our observations more closely resemble the case of S. noursei, in which a strain with a deletion of a nystatin synthesis regulator still produced some antibiotic (24).

Our data show that not only is BldD important for erythromycin biosynthesis, it is also necessary for morphological differentiation. We report the discovery of a developmental transcription factor that directly activates expression of the enzymes of an antibiotic biosynthetic pathway. The findings here provide a starting point for understanding the regulation of erythromycin biosynthesis. The complex mechanisms that generate antibiotics in actinobacteria involve factors such as small signaling molecules (25) and hierarchical of transriptional proteins (26). Attempts to understand how proteins such as BldD work with these factors should promote progress toward new strategies for strain improvement. The same efforts should reveal further how bacteria connect the synthesis of small molecules to their morphogenesis.

**Materials and Methods**

**Bacterial Strains and Media.** S. erythraea WT NRRL2338 and OVP strain K41–135 were obtained from the American Type Culture Collection and Kosan Biosciences, respectively. All S. erythraea strains were grown and stored as described in ref. 20. E. coli strains XL1-Blue and One Shot TOP10 (Invitrogen) were used for DNA cloning. E. coli BL21(DE3) (Novagen) was used for heterologous production of BldD.

**Generation of Fluorescent Probes for the Promoters of the ery Cluster.** Promoter fragments were generated by PCR with Taq polymerase by using the primers listed in Table S1. The PCR products were purified and concentrated by gel
purification and end-labeled with cyanine-3-DCP. The end-labeling reaction consisted of 1 μg PCR product, 1X TdT Buffer, 0.25 mM CoCl_2, 30 units of TdT (NEB), and 1 nmol of cyanine-3-DCP (Perkin-Elmer) in a final volume of 20 μl. The reaction was incubated at 37°C for 1 h and then heat inactivated at 70°C for 10 min. Unincorporated fluorescent nucleotides were removed by washing the reactions twice with 400 μl of TE (10 mM Tris, 1 mM EDTA (pH 7.5)) on Microcon YM-10 filters (Amicon). The labeled probe was concentrated to a final volume of 20 μl.

Protein Preparation and Quantification. S. erythraea strains were grown in RS medium for protein harvesting experiments. Cells were harvested by centrifugation and cell pellets were resuspended in TA Buffer (14) with 50 mM NaCl and Complete protease inhibitor mixture (Roche). Cells were lysed by sonication and cell pellets were resuspended in TA Buffer (14) with 50 mM NaCl and 10–50 μg of crude or partially purified protein. Reactions were incubated on ice for 10 min and then run on a BSA TBE gel (Bio-Rad) buffered in 0.5X TBE (45 mM Tris–borate, 1 mM EDTA) at 20 mA. Gels were imaged on a Typhoon 9410 Variable Mode Imager (GE Healthcare).

Electrophoretic Mobility Shift Assays. The binding reaction consisted of 10 mM Tris (pH 7.5), 5 mM MgCl_2, 50 mM EDTA, 60 mM KCl, 10 mM DTT, 10% glycerol, 1 μg of poly dIdC, 1 μl of labeled probe (~50 ng), and 10–50 μg of crude or partially purified protein. Reactions were incubated on ice for 10 min and then run on a BSA TBE gel (Bio-Rad) buffered in 0.5X TBE (45 mM Tris–borate, 1 mM EDTA) at 20 mA. Gels were imaged on a Typhoon 9410 Variable Mode Imager (GE Healthcare).

Production of BldD in E. coli. The bldD gene was amplified with Ncol and BamHI restriction sites from the S. erythraea overproducer genome by using primers 5'-CCATGGGCAGCTACCGGAAC-3' and 5'-GGATCCCTACTCTCCTCCCGGGCGACCGGACCCGCGGGCC-3' with Pfu polymerase (Stratagene) and cloned into pET15b (Novagen). pET15b/bldD was digested with Ndel and EcoRI and the resulting 500 bp fragment was purified and ligated into pET21b (Novagen). The vector was introduced by transformation into E. coli BL21(DE3) for protein production. Fifty milliliters of LB containing 100 μg/ml carbenicillin was inoculated with a single BL21(DE3) colony. Cultures were grown at 30°C to an OD_600 of ~0.4–0.6. The inducer isopropyl β-D-thiogalactoside was added to each culture to a final concentration of 0.4 mM. Cultures were induced for 3 h before lysis. Cells were harvested by centrifugation and washed with chilled TE (50 mM Tris (pH 7.5), 1 mM EDTA). The pellets were resuspended in Buffer D (200 mM sodium phosphate, 200 mM NaCl, 2.5 mM DTT, 1.5 mM benzamidine, 2.5 mM EDTA, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 30% glycerol, pH 7.1) and sonicated. Lysates were harvested by centrifugation.

Purification of Recombinant BldD. E. coli BL21(DE3) containing pET15b/bldD was grown for induction and harvested as described in paragraph 6 of Materials and Methods. We used the cleavage site for thrombin adjacent to the histidine tag in the vector to remove the tag after the protein had been purified with a nickel column. The thrombin was removed by using p-aminobenzenesulfonic acid. BldD was purified to almost single band purity in SDS-PAGE. See SI Materials and Methods for more details.

DnaseI Footprinting of eryBVI. The DnaseI footprinting experiment was performed as described in ref. 30. See SI Materials and Methods for more details.

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Supporting Information

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SI Materials and Methods

Purification of BldD from S. erythraea. BldD was purified from crude protein extracts from ten liters of S. erythraea overproducer culture. All binding activity was monitored using gel shifts with fluorescently labeled eryBVlp probe. Tryptic soy broth seed cultures were inoculated from mycelial banks and grown for 2 days at 30°C. R5 (1) cultures were inoculated with the seed cultures to an OD_{600} of 0.1. The R5 cultures were grown in 1-liter batches, separated as 100-ml cultures per 500-ml shake flask, for ~20 h (OD_{600} = 1.5–4.5). Cultures were harvested by centrifugation at 2,000 × g for 15 min on a J-10 rotor. Cell pellets were washed twice with 50 mM NaCl TA Buffer [10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.1% (vol/vol) Triton X-100, 10% glycerol] (2), frozen on dry ice, and then stored at −20°C until all cultures were grown. Cell pellets were resuspended in 50 mM NaCl TA Buffer with Complete protease inhibitor (Roche) (final volume of 310 ml) then lysed by sonication (Branson Digital Sonifier 450). Cell lysates were centrifuged at 13,000 × g for 1 h at 4°C to remove cell debris.

The supernatant was precipitated in three fractions: 0–40%, 40–60%, and 60–75%. Precipitated proteins were harvested by centrifugation and resuspended in 20 mM NaCl TA buffer minus Triton X-100 (TAm). Fractions were dialyzed overnight against 20 mM TAm. Active fractions 40–60% and 60–75% were pooled together and passed through a 0.45-µm filter before the FPLC purification steps. The protein sample was first loaded at a rate of 3 ml/min onto a 65-ml DEAE Sepharose Fast Flow Column (XK26, Amersham) equilibrated in 20 mM NaCl TAm. Bound proteins were eluted from the DEAE column using a linear NaCl gradient of 20–500 mM over 180 ml of TAm buffer. Fractions containing eryBVlp binding activity were pooled together and dialyzed against 20 mM NaCl TAm. The active fractions were next loaded at 2 ml/min onto a 22-ml Hi-trap Heparin HP column (XK16, Amersham) equilibrated in 20 mM NaCl TAm. Bound proteins were eluted from the heparin column using a linear gradient of 20–500 mM NaCl TAm over 50 ml. Active fractions were pooled and dialyzed against 20 mM NaCl TAm.

The highly active fractions were then purified by affinity chromatography. The fractions were allowed to bind separately to the eryBVlp beads. Eight hundred microliters of the pooled heparin fractions were incubated with 200 µl of affinity beads for 45 min on a rotator at 4°C. The beads were washed with 20 mM NaCl NaCl TAm buffer to remove unbound protein. Next, the beads were washed three times with 50 µg poly(dI-dC), a nonspecific DNA competitor, in 20 mM NaCl TAm buffer. Bound protein was eluted off the beads in three 250-µl fractions (200 mM, 400 mM, and 800 mM NaCl TA buffer). Beads were regenerated by washing with 1 M NaCl TAm. Two hundred microliters of each salt fraction was precipitated with trichloroacetic acid for protein visualization on SDS-PAGE.

Preparation of eryBVlp Affinity Matrices. Biotinylated eryBVlp was generated by 96-well PCR (9.6 ml total volume) using Taq polymerase for each probe. S. erythraea overproducer genomic DNA was used as the template in the PCR reactions. For eryBVlp, a biotinylated version of the eryBVlp-reverse primer with the normal eryBVlp-forward primer was used to generate the fragments. To remove unincorporated biotinylated primers, the pooled PCR reactions were purified on a 1-ml monoQ HR5/5 column. Fractions were assayed by gel electrophoresis and fractions containing PCR products were pooled together. Purified DNA was concentrated and desalted using Microcon YM-10 filters (Amicon) to a final volume of 2 ml for each biotinylated DNA probe.

The biotinylated PCR products were linked to streptavidin (SA) magnetic particles (Roche). SA beads were first equilibrated in 20 mM TAm buffer. In the binding reaction, ~120 µl of purified PCR product diluted to 1 ml with 20 mM NaCl TAm buffer was added to 200 µl of beads for each probe. Reactions were incubated at 4°C for 1 h on a rotator. Binding efficiency was checked by gel electrophoresis of pre- and postbinding supernatants. Beads were washed with 20 mM NaCl TAm buffer to remove excess biotinylated PCR product.

Mass Spectrometry Fingerprinting and de novo Sequencing. The 18-kDa protein candidate was excised from a SDS-PAGE gel and submitted for mass spectrometry analysis at the Protein and Nucleic Acid facility at Stanford University. The protein was digested with trypsin and analyzed by MALDI-MS for mass fingerprinting. Mass spectrometry de novo sequencing using CAF reagent (Amersham) also provided amino acid sequences (3).

Cloning of bldD. A ligation mediated PCR (LMPCR) protocol developed by K. Fowler (4) was used to obtain the bldD DNA sequence from the S. erythraea overproducer genome. bldD-specific primers were designed using the amino acid sequence HGVEQK from peptide 2 based on probable codon usage. In the LMPCR reactions, 0.5 µg of S. erythraea overproducer genomic DNA was digested with EagI. The digested DNA was ligated to adapter DNA which was made by annealing primers UNIVS-AD1 (5'-GACTCGCGAATTCCGACAGTTGA-3') and EAGI-AD2 (5'-GGCCTCAACTGTCG-3'). One microliter of the ligation reaction was used as a template for PCR. UNIVS-AD1 was paired with the various HGVEQK primers to generate a PCR product.

The first round of LMPCR reactions yielded a 250-bp PCR product from HGVEQK primers 2, 4, and coel. A reverse primer, HGVEQK-REV1 (5'-GTCGCCACGTTCATAGGGACC-3') was designed from the 250-bp product and was used to amplify sequence upstream of the 250-bp product. To extract sequence downstream of the 250-bp product (i.e., past the EagI site), the primer 250bpEagI (5'-GCAACAGCTCCGGCGCGCC-3') was designed at the end of the 250-bp sequence. Overproducer genomic DNA was partially digested with EagI for 5 min to maintain nondigested bldD in the DNA population. LMPCR was performed on this digested DNA with 250bpEagI. The reaction yielded a range of PCR products, which included a major 300-bp band. This region was gel purifed and cloned into the PCR-BluntII-TOPO plasmid. Cloned inserts were then sequenced. From the sequencing reactions, one clone sequence confirmed three of the other peptide sequences determined by mass spectrometry and thus completed the bldD DNA sequence.

Gene Deletion and Complementation of bldD. The REDIRECT protocol for S. coelicolor targeted gene replacement was used (5), with slight modifications at the conjugation step (O. Lazos, personal communication). Primers in supporting information (SI) Table S2 were used to generate the apramycin resistance cassette. The S. erythraea cosmID p2084 containing the bldD gene was used to generate the disrupted bldD cosmid with the apramycin resistance cassette. Subsequently, the cosmid was conjugated into AML315–638 with Escherichia coli ET12567/pUZ8002 and plated onto R5 agar with 50 µg/ml nalidixic acid. AML-315 was grown in 5 ml M79 medium (6) for several days

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until dense and then 300 µl of the seed culture was reinoculated into 5 ml of fresh M79. Five hundred microliters of the mycelial and 500 µl of E. coli were used for each conjugation onto soy flour mannitol plates. The next day, the conjugation plates were overlayed with 3 ml of semi-SFM (1:5 SFM:H2O vol/vol) containing 1.5 mg of apramycin and 1 mg of nalidixic acid. The plates were incubated at 30°C until colonies became visible and were restreaked onto M1 plates with apramycin. The colonies obtained were checked with Southern hybridization to confirm that a gene deletion had occurred.

To reintroduce bldD into the knockout (KO) strain, primers 5'-gaattcGACGCCACGGTGGAAACC-3' and 5'-ggtaccT-CACTCCTCCCGGGCC-3' were used to amplify bldD from the S. erythraea genomic DNA and then cloned into the EcoRI/KpnI sites of pKOS460–18 to generate pKOS460–18::bldD. Subsequently, this plasmid was reintroduced into the KO strain via conjugations as described above.

**Purification of Recombinant BldD.** The strain E.coli BL21(DE3) with pET15b/bldD was grown in 100 ml of LB with carbenicillin (50 µg/ml) at 37°C for 4 h until the culture reached a density of OD600 ≈ 0.6. Then, 0.4 mM isopropyl-β-D-thiogalactopyranoside was added to induce the synthesis of BldD. After the culture grew for 3 additional hours at 37°C, cells were harvested. Upon centrifugation, the cells were washed twice in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8) before resuspending in lysis buffer. The cells were sonicated and the supernatant was incubated with Ni-NTA resin (Qiagen) at 4°C for 1 h. The beads were then spun down and washed twice with lysis buffer, twice with wash buffer (50 mM NaH2PO4, 300 mM NaCl, 40 mM imidazole, pH 8), and finally with PBS with 10 mM imidazole. The beads were resuspended in PBS and incubated with 12 units of bovine thrombin (MP Biomedical) for 4 h at room temperature. p-aminobenzamidine agarose (Sigma) was added to the supernatant for 30 min to remove the thrombin. The purified BldD was quantified at OD280 by using a calculated molar absorption coefficient (7).

**DNAaseI Footprinting of eryBV1.** The DNAaseI footprinting experiment was performed as described in ref. 8. Briefly, to generate the eryBV1p DNA fragment from S. erythraea genomic DNA, the primers had sequences similar to those used in EMSAs, except the 5’ end was labeled with 6-FAM on the template strand and NED (Applied Biosystems) on the nontemplate strand. The PCR product was purified by gel electrophoresis and quantified. The binding reaction resembled that used for EMSAs, except that EDTA, which inhibits DNAaseI, was omitted. A sample of BldD (54 µM) was incubated with the probe on ice for 25–30 min, after which various concentrations of DNase I (Invitrogen) were added. Based on the results of optimization experiments using various concentrations, 0.1 units of DNase I was added to the reaction mixture and incubated for 5 min at 25°C. The reaction was incubated for 2 min at room temperature and terminated by adding 1 µl of 25 mM EDTA and heating at 65°C for 10 min. The reaction was passed through the Nucleotide Removal Kit (Qiagen) before drying the sample and analysis on a 3730 DNA Analyzer (Applied Biosystems). Nondigested DNA was used for sequencing reactions with the 5’ FAM-labeled forward primer and a Thermo Sequenase dye primer manual cycle sequencing kit (USB Corp.). The digested DNA and sequencing reaction products were analyzed with a 3730 DNA analyzer as described in ref. 8. The sequences were then analyzed with GeneMarker (SoftGenetics) software to determine the protected sequences in the DNase I digestion map.

Fig. S1. EMSAs with promoters of the ery cluster and lysates of *S. erythraea*. EMSAs for the three regions of promoters, designated eryBI-BIII, eryCI-ermE, and eryK. Each probe has lanes b–f. Lanes: DNA ladder (a); probe only (b); probe + WT 22-hr lysate (c); probe + WT 65-hr lysate (d); probe + overproducer 22-hr lysate (e); and probe + overproducer 65-hr lysate (f).
Fig. S2. Optical density of the wild-type (WT, black diamonds) and overproducer (OVP, open squares) strains grown in a liquid, rich medium.
Fig. S3. DNA and amino acid sequence of bldD. Black, blue, and red text represent the upstream region, amino acid sequence, and DNA sequence of bldD, respectively. Gray boxes denote peptide sequences predicted by mass spectrometry.
Fig. S4. Alignment of eryBVI DNase I footprint to sequencing data. Boxed region denotes region protected by BldD. The electrophoregrams were aligned using GeneMarker from SoftGenetics.
Fig. 55. Binding curves of BldD against promoter regions of bldD (A), eryBVI (B), eryAI-IV (C), eryBI-BIII (D), eryCI ermE (E), and eryK (F). The black diamonds, black triangles, and open diamonds represent data from three independent experiments.
Phenotypes of *S. erythraea* lacking bldD. Strains were grown on two solid media (SFM and R5). WT, Wild type *S. erythraea*; ΔbldD, WT strain with bldD deleted; ΔbldD::bldD, ΔbldD strain with bldD integrated on a vector next to the ery cluster (see text for details); and ΔbldD::empty vector, ΔbldD strain with an empty vector integrated next to the ery cluster, which served as a negative control.
Table S1. Primers to amplify EMSAs probes

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Primer 1</th>
<th>Primer 2</th>
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<tbody>
<tr>
<td>eryCl-ermE</td>
<td>GTGCTGCCGATCTGGTGTCGTGG</td>
<td>CGGACCGGAGTTCGAGGTACGC</td>
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<tr>
<td>eryBII-III</td>
<td>CGACACGGACTCGAGGTACGG</td>
<td>GATACGGCCGACGACGACGC</td>
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<tr>
<td>eryAI-IV</td>
<td>CCCGGCTCCCGGACGAGCC</td>
<td>CACGGACGAAGATCTCCCAGG</td>
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<td>eryBIIIp</td>
<td>GCCGAGCAGAAGACGCTTGGC</td>
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<tr>
<td>eryK</td>
<td>GACCGGATCCGGCTCCCAGAG</td>
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Table S2. Primers to delete bldD from S. erythraea

<table>
<thead>
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<th>Primers</th>
</tr>
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<tbody>
<tr>
<td>TCGGCTGACGGGGCGAATGAAACGGGAGAAACGACATGattccgggatccgtrgacc</td>
</tr>
<tr>
<td>GGGCCACCCGATCGGGGTGGGCGTTCCGCGTTTGGTTGTCGTCatgtagctggagctgcttc</td>
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