Knockout of the Erythromycin Biosynthetic Cluster Gene, *eryBI*, Blocks Isoflavone Glucoside Bioconversion during Erythromycin Fermentations in *Aeromicrobium erythreum* but Not in *Saccharopolyspora erythraea*†

Andrew R. Reeves,† Ramya Seshadri,‡ Igor A. Brikun,§ William H. Cernota, Melissa C. Gonzalez, and J. Mark Weber*

Fermalogic, Inc., 2201 West Campbell Park Drive, Chicago, Illinois 60612

Received 30 July 2008/Accepted 25 September 2008

Isoflavone glucosides are valuable nutraceutical compounds and are present in commercial fermentations, such as the erythromycin fermentation, as constituents of the soy flour in the growth medium. The purpose of this study was to develop a method for recovery of the isoflavone glucosides as value-added coproducts at the end of either *Saccharopolyspora erythraea* or *Aeromicrobium erythreum* fermentation. Because the first step in isoilavone metabolism was known to be the conversion of isoflavone glucosides to aglycones by a β-glucosidase, we chose to knock out the only β-glucosidase gene known at the start of the study, *eryBI*, to see what effect this had on metabolism of isoflavone glucosides in each organism. In the unicellular erythromycin producer *A. erythreum*, knockout of *eryBI* was sufficient to block the conversion of isoflavone glucosides to aglycones. In *S. erythraea*, knockout of *eryBI* had no effect on this reaction, suggesting that other β-glucosidases are present. Erythromycin production was not significantly affected in either strain as a result of the *eryBI* knockout. This study showed that isoflavone metabolism could be blocked in *A. erythreum* by *eryBI* knockout but that *eryBI* knockout was not sufficient to block isoilavone metabolism in *S. erythraea*.

Many industrial fermentations use soy flour as a growth medium component. Recovery of the isoflavones from this soy flour represents an untapped source of value-added fermentation coproducts. For over 10 years, the isoflavones genistein and daidzein have been intensively investigated to determine their health benefits (1, 7, 9) and have been added as nutraceutical ingredients to food and health products. Erythromycin fermentation was the first pharmaceutical fermentation to be investigated as a model system for isoflavone recovery (5). Erythromycin is a bulk fermentation product used in its natural form and in various salt forms as an antibiotic. It is also used as a chemical intermediate in the production of other widely prescribed antibiotics, including azithromycin, clarithromycin, dirithromycin, and roxithromycin. Hundreds of thousands of tons of erythromycin are produced each year. The amount of isoflavone coproduced could be as much as 0.5 to 1% of the amount of erythromycin produced, and since the value of isoflavone coproduced could be as much as 0.5 to 1% of the amount of erythromycin produced, an significant economic gain could be obtained. In a previous study (5), it was found that the main technical problem with recovering isoflavones as coproducts was their bioconversion and degradation by the fermentation organism.

Therefore, in order to make isoflavone recovery technically possible, a method for blocking isoflavone metabolism was sought.

Previously, we reported that in the course of erythromycin fermentation, *Saccharopolyspora erythraea* enzymatically hydrolyzes glucose from the isoflavone glucosides genistin and daidzin. However, the β-glucosidase used in this biotransformation was not identified or characterized (5). Interestingly, the erythromycin biosynthetic gene cluster, which has been extensively studied and characterized in *S. erythraea* (for a review, see reference 12), contains a β-glucosidase gene. This gene was referred to originally as *eryBI* (18) and later was renamed *orf2* because of its lack of involvement in erythromycin biosynthesis in *S. erythraea* (4); more recently, it has been suggested that the designation should be changed to *eryBI* (12).

*eryBI* was originally defined as a genetic locus immediately downstream of the *ermE* gene in *S. erythraea*. Its map location corresponded to the insertion site of pMW55-27 via the cloned DNA fragment BI-27 (Fig. 1) (18). Insertion of pMW55-27 into the chromosome resulted in a block in erythromycin A production and accumulation of the erythromycin precursor erythronolide B, giving the mutant the EryB phenotype (17). The *eryBI*-27 mutation, however, was created and analyzed before the DNA sequence of this region was available. Later, after the sequence was published (4), it was seen that pMW55-27 inserted close to, or possibly overlapped, the 3′ terminus of the open reading frame in this region. Why plasmid pMW55-27 conferred the EryB phenotype after insertion into this region of *eryBI* is unknown. It would have been expected to simply confer a wild-type phenotype, like other mutations in this gene (Fig. 1). Nevertheless, because of this
result, the gene downstream of *ermE* became known as *eryBI* despite its lack of involvement in erythromycin biosynthesis under the conditions tested so far.

A new theory about a possible function for EryBI was proposed when EryBI was found to be homologous to OleR (61% identity to the OleR amino acid sequence) encoded by a gene in the oleandomycin gene cluster. OleR functions as a component of the oleandomycin self-resistance mechanism in *Streptomyces antibioticus* (11). Its \(\beta\)-glucosidase activity is specific to the oleandomycin glucoside made by *S. antibioticus*. It was hypothesized that at one time, EryBI may have played a role analogous to that of OleR in *S. erythraea*. However, unlike oleR, the *eryBI* gene was thought to have become nonfunctional because it was redundant after *S. erythraea* acquired a second, more effective erythromycin resistance gene, *ermE* (11). The experimental data at the time were consistent with this theory and with the conclusion that EryBI is a nonfunctional gene.

When this study began, there was no evidence of linkage between *eryBI* and isoflavone metabolism, although the possibility of a connection had been discussed (4). To address this possibility, experiments were performed using two different erythromycin-producing organisms, *S. erythraea* and *Aeromicrobium erythreum* (2), in order to test for \(\beta\)-glucosidase activity against isoflavone glucosides. The hypothesis was that if the *eryBI* gene was found to be involved in the conversion of isoflavone glucosides, then inactivation of *eryBI* could make the recovery of isoflavone glucoside coproducts from erythromycin fermentation possible without interfering with erythromycin production.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The general materials and methods used for *Escherichia coli* and *Actinomycetes* were described by Sambrook et al. (16) and Kieser et al. (6), respectively. The bacterial strains and plasmids used in this study are described in Table 1. We used *S. erythraea* FL2267, a “white” strain and a derivative of ATCC 11635 (15); also, we used *A. erythreum* wild-type strain FL262 (= NRRL B-3381), which has been described previously (14). We also used *S. erythraea* strain FL1347, a red variant strain which is a spontaneous, red-pigmented derivative of *S. erythraea* white strain ATCC 11635 and produces small amounts of erythromycin. FL1347 transforms with plasmid DNA at a higher efficiency than FL2267. Spores of *S. erythraea* white strains were produced on E20A agar (13). *A. erythreum* was maintained on 2× YT agar plates or as

---

**FIG. 1.** Structure of erythromycin A and map of the *eryBI* region of the *S. erythraea* erythromycin biosynthetic gene cluster. The scale for the map positions is based on the convention used for the genomic sequence (8). Restriction sites were mapped using the genomic sequence information; however, the restriction site numbering system is that of Weber et al. (18). The pFL2191-D and pFL2191-U lines represent PCR fragments generated during construction of plasmid pFL2191 used for deletion of *eryBI* in *S. erythraea*. The abbreviations for the restriction sites at the PCR product ends are as follows: S, SstI; E, EcoRI; and H, HindIII. The pFL2168 line represents the equivalent homologous *A. erythreum* DNA fragment generated by PCR from an *A. erythreum* DNA template and cloned to create pFL2168 for knockout of the *eryBI* gene of *A. erythreum*. Double asterisks indicate the *eryBI* mutations described by Gaisser et al. (4); single asterisks indicate the mutations described by Weber et al. (18). The colored boxes indicate the possible conserved domains detected by BLASTP analysis of the EryBI amino acid sequence (September 2008). The numbers above the domain structure refer to the amino acid sequence of the EryBI protein of *S. erythraea*. The gray box indicates the equivalent \(\beta\)-glucosidase group in oleandomycin that is glucosylated by the oleandomycin glucosyltransferase and then hydrolyzed by OleR, both of which are components of the oleandomycin resistance mechanism of *S. antibioticus* (11).
glycerol stocks at -80°C. OFM1 (which contains 22 g/liter soy flour) and CFR medium have been described previously (15); modified SCM (fermentation) broth has also been described previously (14). When fermentations were performed in SCM supplemented with soy flour, 22 g/liter of soy flour was added.

Plasmids used to create eryBI knockouts in *A. erythreum* and *S. erythraea*. Plasmid pFL2168, which was used to create the eryBI knockout in *A. erythreum*, was constructed from an internal 593-bp eryBI PCR fragment (Fig. 1) cloned into pFL2092 (14). The *A. erythreum* chromosomal DNA template was used with primers eryBIAeF1 (5'-GTCGAGCTCAGGACATCTGGTCACGCTC-3') and eryBIAeR1 (5'-GTCGAGCTCAGGACATCTGGTCACGCTC-3'). The PCR product was ligated into pGem-T Easy (Promega, Madison, WI) and then electroporated into *E. coli* DH5α-e and plated on 2× YT agar (16) containing ampicillin and the indicator X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plasmids were isolated from colorless transformants and analyzed by using restriction analysis, and the DNA sequence of the insert was determined to confirm its identity. The resulting intermediate construct, pFL2164, was digested with EcoRI to release the eryBI fragment, which was ligated to EcoRI-digested pFL2092. Ligation mixtures were electroporated into *E. coli* DH5α-e, and recombinant plasmids were confirmed by restriction digestion and DNA sequencing. One plasmid, pFL2166, was used to transform wild-type *A. erythreum* using the method of Reeves et al. (14).

Plasmid pFL2191, which was used for generation of *S. erythraea* eryBI deletion strain FL2316, was constructed by using four components. The first component was pFL8 (13) digested with SacI and HindIII. The second and third components were obtained from PCR products amplified from two noncontiguous chromosomal regions (pFL2191-D, downstream of eryBI; and pFL2191-U, upstream of eryBI [Fig. 1]). The primers used to generate a PCR product downstream of eryBI in pFL2191-D were CISEFD-1 (5'-GTCGACGTTGATCGGCCAGCATTCCTC-3') and ermERV-1 (5'-GTCGACGTTGATCGGCCAGCATTCCTC-3). The primers used to generate a PCR product upstream of eryBI were BIPROFD-1 (5'-GTCGACGTTGATCGGCCAGCATTCCTC-3') and FSRV-1 (5'-GTCGACGTTGATCGGCCAGCATTCCTC-3'). The fourth component used for ligation was the kanamycin resistance gene from pUC4K (Pharmacia Biosciences, Piscataway, NJ), which was released by digestion with EcoRI. The four components were ligated together and electroporated into *E. coli* cells with selection for kanamycin-resistant clones on 2× YT plates. One plasmid, pFL2191-1 (Table 1), was used to transform *S. erythraea* FL2267 (white) and FL1347 (red) protoplasts as described by Reeves et al. (15) with selection for apramycin, kanamycin, and thiostrepton resistance.

**TABLE 1.** *S. erythraea* and *A. erythreum* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Plasmid insert or strain description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFL8</td>
<td><em>S. erythraea</em> parent plasmid used for generation of integration vectors; carries genes for ampicillin and thiostrepton resistance</td>
<td>13</td>
</tr>
<tr>
<td>pFL2092</td>
<td><em>A. erythreum</em> vector used for integrating DNA sequences via homologous recombination; contains the thiostrepton resistance gene from pFL487 cloned into the EcoRI and KpnI sites of pFL2082; carries genes for ampicillin, kanamycin, and thiostrepton resistance</td>
<td>14</td>
</tr>
<tr>
<td>pFL2168</td>
<td><em>A. erythreum</em> integration vector containing a 593-bp eryBI internal fragment cloned into the unique EcoRI site of pFL2092; used to generate a knockout in <em>eryBI</em> by single-crossover insertion; carries genes for ampicillin, kanamycin, and thiostrepton resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pFL2191</td>
<td><em>S. erythraea</em> gene replacement vector used to delete <em>eryBI</em> and insert <em>aphI</em> (kanamycin resistance gene) in its place</td>
<td>15</td>
</tr>
</tbody>
</table>

**Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1347</td>
<td>Red variant (less erythromycin produced) derivative of <em>S. erythraea</em> ATCC 11635</td>
<td>13</td>
</tr>
<tr>
<td>FL2296</td>
<td>Derivative of <em>S. erythraea</em> FL1347 with <em>eryBI</em> deleted and replaced by the <em>aphI</em> (kanamycin resistance) gene using pFL2191</td>
<td>This study</td>
</tr>
<tr>
<td>FL2267</td>
<td>White derivative of <em>S. erythraea</em> ATCC 11635; wild-type white strain; used as host strain for transformation</td>
<td>15</td>
</tr>
<tr>
<td>FL2316</td>
<td>Derivative of <em>S. erythraea</em> FL2267 with the <em>eryBI</em> gene deleted and replaced by the <em>aphI</em> (kanamycin resistance) gene using pFL2191</td>
<td>This study</td>
</tr>
<tr>
<td>FL262</td>
<td>Wild-type <em>A. erythreum</em> strain; NRRL-B-3381</td>
<td>14</td>
</tr>
<tr>
<td>FL264</td>
<td>Derivative of <em>A. erythreum</em> FL262 having a knockout in <em>eryBI</em> due to single-crossover insertion of pFL2168</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α-e</td>
<td>Host strain used for electroporation Invitrogen, Carlsbad, CA</td>
<td></td>
</tr>
</tbody>
</table>

**Confirmation of gene knockouts in *eryBI* mutants by PCR.** PCR primers were designed so that a 1.879-kb region was amplified in *A. erythreum* eryBI mutant FL2264 but was missing in wild-type strain FL262. Based on the predicted integration of pFL2168, the region amplified 278 bp of *ermA* (2), 1.513 kb of the adjacent region in *eryBI*, and 88 bp of pFL2092. The primers used were forward primer ermAeF1 (5'-CTCGAAGATCGACGCTGCAAGTCGTGCTCCGTCATCC-3') and reverse primer 2092BIR1 (5'-AGCTGCGAAAAGGGGAGATCTG-3'). The reverse primer was located 88 bp upstream of the EcoRI site of pUC19-based (pFL2092) plasmids. To confirm that there was gene replacement in pFL2191 and that there was insertion of the kanamycin resistance gene in *S. erythraea* FL2316, PCR primers were designed to amplify a 1.549-kb region spanning the inserted kanamycin resistance gene and surrounding upstream (*ermE*) and downstream (*eryBIII*) sequences. The primers used were forward primer ermF1 (5'-GACCAGAATCGCCAGACGAG-3') and reverse primer ermBIIIIR1 (5'-GCGATAGCGGATTGCTTCGTT-3').

**Shake flask fermentations.** For *A. erythreum* seed preparation, 25 ml of MScM in 250-ml flasks was inoculated with 25 µl of a frozen glycerol stock culture and incubated at 380 circular rpm, 65% relative humidity, and 32.5°C for 24 h. For FL264, a thiostrepton-resistant *eryBI* mutant strain, thiostrepton was added to MScM at a concentration of 3 µg/ml. Twenty-five milliliters of MScM per 250-ml shake flask was used for fermentations. A 1.25-ml portion of an appropriate seed culture was used to inoculate a fermentation flask, and fermentation was performed under the same growth conditions that were used for the seed cultures. Broth samples (0.75 ml) were taken at intervals and stored at -80°C for later analysis.

*S. erythraea* shake flask fermentation was performed at 380 circular rpm in unbaffled 250-ml Erlenmeyer flasks with milk filter closures. The flasks were incubated at 32.5°C and 65% humidity on an Infors Multitron shaker having a 1-in. circular displacement. Seed cultures were inoculated using fresh spores prepared from E20A agar plates; the 250-ml shake flask each contained 25 ml of CCM1 broth (15) and were incubated on the same shaker and under the same growth conditions that were used for the fermentations. Fermentation preparations were inoculated with 1.25 ml of a seed culture in late logarithmic growth phase (30 to 45 h) and contained 25 ml of OFM1 broth. The fermentation cultures were grown for 5 days, and the final volumes were corrected for evaporation by addition of water before the cultures were analyzed further.

**Processing of fermentation samples for bioassays, TLC, and HPLC.** Cells were removed from broth samples by centrifugation, and each supernatant was passed through a 0.45-µm filter. A 50-µl portion of the filtered supernatant was removed for bioassay analysis, and the remainder of the supernatant was used for...
further analysis by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The remaining supernatant (700 μl) was extracted with 500 μl of ethyl acetate. The extract was concentrated to dryness, and the extract was then resuspended in 100 μl of acetonitrile. A 50-μl portion of the acetonitrile solution was used for HPLC analysis, 5 μl was used for analysis of isoflavones by TLC, and 10 μl was used for analysis of erythromycin by TLC.

Bioassay. Bioassays were performed to determine the erythromycin yields in culture broth, and the methods used have been described previously (14).

TLC. For analysis of isoflavones, 5-μl portions of the acetonitrile extracts along with standards (daidzin, genistin, and daidzein plus genistein) were applied to TLC plates, and the plates were developed using chloroform-methanol-acetic acid (10:1:1). Spots were visualized by fluorescence quenching at 254 nm. The plates were photographed while they were being exposed to the UV light. For analysis of erythromycin, 10-μl portions of the acetonitrile extracts along with 4 μl of erythromycin standards were applied to TLC plates, and the plates were developed using isopropyl ether-methanol-ammonium hydroxide (75:35:1). Spots were visualized by spraying the plates with a mixture of p-anisaldehyde, sulfuric acid, and ethanol (3:3:27) and then heating the freshly sprayed plates at 100°C for 5 min.

HPLC analysis. The analysis of isoflavones by HPLC was performed using an Hitachi Elite LaChrom system composed of a quaternary pump, an autosampler, a UV detector, and LaChrom software. The column used was an Alltech Previcil C18 column (5 μm; length, 250 mm; inside diameter, 4.6 mm) at 30°C. UV detection was performed at 265 nm. The injection volume was 50 μl. The mobile phase was composed of solvent A (water, 1% formic acid) and solvent B (acetonitrile, 1% formic acid), and a flow rate of 1.0 ml/min was used. Sample elution was performed by using a 25 to 50% solvent B gradient over 20 min. Linearity in the range from 0.1 to 5 μg/ml for all standards in ethanol was established, and a standard curve was used to calculate the concentrations for fermentation samples based on the peak areas.

Chemicals, biochemicals, and reagents. Daidzin and genistin standards were obtained from LC Labs (Woburn, MA). Daidzein, genistein, apigenin-7-O-glucoside, and naringenin-7-O-glucoside were obtained from Indofine Chemical Company (Hillsborough, NJ). For A. erythreum fermentations involving spiking of pure flavonoid glucosides the compounds were added to MSCM at a final concentration of 25 μg/ml.

RESULTS

In A. erythreum, eryBI knockout blocks conversion of isoflavone glucosides. The eryBI gene of A. erythreum was knocked out by single-crossover insertion of plasmid pFL2168 (Fig. 1)
to create strain FL2264. The levels of isoflavone glucosides and aglycones were determined by HPLC over the course of 3 days of fermentation in MSCM containing soy flour, and the values obtained for parent strain FL262 and eryBI knockout strain FL2264 were compared.

Soon after inoculation, parent strain FL262 showed conversion of the isoflavone glucosides genistin and daidzin (Fig. 2A). The concentration of isoflavone glucosides dropped from 25 μg/ml at zero time to undetectable levels by 24 h. Over the same 24-h time period the concentration of the isoflavone aglycones genistein and daidzin in the medium increased from 2 μg/ml to 20 to 25 μg/ml. Once formed, the isoflavone aglycones were maintained stably for the remainder of the fermentation, and no other isoflavone biotransformation products were produced.

By contrast, in A. erythreum strain FL2264 carrying the eryBI knockout mutation, the isoflavone glucoside concentrations did not drop as they did in the parent strain; instead, they were stably maintained in the fermentation broth in the range from 20 to 25 μg/ml (Fig. 2B). No other isoflavone biotransformation products appeared to form during the eryBI knockout strain fermentation, as visualized by TLC analysis (Fig. 2E).

In S. erythraea, eryBI knockout did not block conversion of isoflavone glucosides. An eryBI knockout strain of S. erythraea FL2267 was constructed by double-crossover replacement of the eryBI gene with the kanamycin resistance gene using plasmid pFL2191 (Fig. 1) to create strain FL2316. This effectively deleted the entire eryBI gene and replaced it with the kanamycin resistance gene. This eryBI knockout strain was compared directly to parent strain FL2267 using soy flour-based shake flask fermentations with OFM1 (soy) medium.

After inoculation of parent strain FL2267 into the soy-based (OFM1) medium, the concentration of isoflavone glucosides decreased rapidly from 18 μg/ml at the first measurement to less than 5 μg/ml within the first 4 h (Fig. 2C). The rate of bioconversion was significantly higher than the rate in A. erythreum fermentations. During the initial 4-h time period the concentration of isoflavone aglycones increased from 2 μg/ml to 8 to 10 μg/ml. This level was lower than the levels of aglycones reached during the A. erythreum fermentation and was probably a reflection of how rapidly the aglycones were biotransformed further during the fermentation. When strain FL2316 carrying the eryBI knockout mutation was observed under identical conditions, the course of isoflavone metabolism was not noticeably different from the course that was observed with the parent strain (Fig. 2D).

The same experiment was performed with the S. erythraea red variant wild-type strain FL1347 and the corresponding eryBI deletion strain FL2296, also made with pFL2191. Similar results were obtained; that is, no effects on isoflavone processing were seen as a result of the eryBI mutation (data not shown).

**eryBI is not required for erythromycin production in either A. erythreum or S. erythraea.** For A. erythreum, the erythromycin production by parent strain FL262 was compared with the erythromycin production by eryBI knockout strain FL2264 using the modified carbohydrate-based fermentation medium (MSCM) containing soy flour. eryBI knockout strain FL2264 produced 140 ± 15 μg/ml, which was not statistically significantly different from the amount of erythromycin produced by parent strain FL262, 190 ± 50 μg/ml (Fig. 3A).

Erythromycin production by the S. erythraea wild-type strain and the eryBI deletion mutant in a high-production oil-based medium (OFM1) (15) was also determined. Shake flask fermentations performed in triplicate revealed that the average value obtained for parent strain FL2267 was 680 ± 170 μg/ml, compared to the 580 ± 120 μg/ml obtained for eryBI mutant strain FL2316. Therefore, there was no statistically significant difference in erythromycin production between the eryBI mutant and the wild-type strain (Fig. 3A). These results for S. erythraea confirmed results obtained previously (4).

TLC analysis of extracts of broth from both fermentations
Fig. 3B showed that primarily erythromycin A was present. No evidence of accumulation of erythronolide B or any other erythromycin biosynthetic intermediate was seen for either organism.

The same experiment was performed with S. erythraea red variant strain FL1347 and an eryBI deletion derivative of FL1347 (FL2296). FL2296 was constructed by gene replacement using pFL2191. Similar results were obtained; that is, no effect on erythromycin production was seen as a result of the eryBI mutation (data not shown).

In A. erythreum, EryBI can convert other classes of flavonoid glucosides to the aglycones. A. erythreum wild-type strain FL262, A. erythreum eryBI knockout strain FL2264, and S. erythraea red variant strain FL1347 were grown in SCM medium not supplemented with soy flour but supplemented with 25 μg/ml flavonoid glucosides. The results of a TLC analysis are shown in Fig. 4. When daidzein was used as an example, the results showed that the spot corresponding to daidzin (the glucoside form) was the same after the 24-h incubation period for duplicate eryBI mutant strains (FL2264) (Fig. 4, lanes 5 and 6). The spot corresponding to daidzein (the aglycone form) appeared in the extract from wild-type strain FL262 (lanes 3 and 4), indicating that the wild-type strain was capable of completely converting the glucoside daidzin to the aglycone daidzein. The wild-type S. erythraea red variant strain was also able to convert all four glucosides to aglycones but further converted the aglycone to a new spot (spot BP) that was between the glucoside and aglycone spots (S. erythraea white strain FL2267 also performed the same conversion of the aglycone [data not shown]). Identification of this spot as an aglycone conversion product is the subject of another study (unpublished results). The glucoside form of each structure is shown next to the TLC results in Fig. 4.

**DISCUSSION**

In this study we found that in A. erythreum the EryBI β-glucosidase is responsible for the conversion of isoflavone glucosides to the corresponding aglycones during erythromycin fermentation. This was determined by knocking out eryBI by plasmid insertion and observing that this was sufficient to block the isoflavone conversion reaction. Previously, eryBI had no known function in A. erythreum or S. erythraea, although it was suggested, based on its high degree of identity to oleR, that eryBI could be an erythromycin resistance gene that had become nonfunctional when the organism acquired the more effective resistance gene ermE (11). With the results presented here it is now apparent that the eryBI gene is actively ex-
pressed, and its gene product has broad substrate specificity for flavonoid glucosides, which was unexpected for an enzyme encoded in a macrolide biosynthetic gene cluster.

Although it is now apparent that the *eryBI* gene of *A. erythreum* is functional and that the EryBI proteins of both *A. erythreum* and *S. erythraea* show strong evidence of signal peptide activity (unlike the results of a previous analysis [4]) (Fig. 5), it seems unlikely that the conversion of flavonoid glucosides is the only (or the primary) function of *A. erythreum* EryBI, particularly since there does not appear to be an obvious connection between isoflavone metabolism and macrolide antibiotic biosynthesis.

There could be other functions of *eryBI* that have not been found yet or that are associated with a processed form of this protein. The members of the EryBI family of proteins have a multifunctional domain structure (Fig. 1) that, at least in the case of OleR, has been found to retain the oleandomycin glucosidase activity even after extracellular processing from a 87-kDa protein to a 34-kDa protein (10). Also, because *eryBI* homologs are conserved among many macrolide gene clusters, it is possible that the *eryBI* family of genes does play a role in macrolide biosynthesis under conditions that have not been tested yet. For these reasons we suggest a return to the original designation, *eryBI*, until the genes in this family have been more thoroughly studied and their functions are understood better.

In *S. erythraea*, *eryBI* knockout did not block the bioconversion of isoflavone glucosides, leaving open the possibility that other β-glucosidases are responsible or contribute to the metabolism of isoflavone glucosides. Attempts were made to determine whether the *S. erythraea* *eryBI* gene was functional through heterologous expression in *E. coli*, but plasmid clones containing the *eryBI* gene were too unstable in *E. coli* to answer this question (unpublished results). Six other genes encoding β-glucosidases have been identified in the genome of *S. erythraea* (*SACE_1247, SACE_1284, SACE_1589, SACE_4101, SACE_5452, and SACE_6502*) (8), and two of these genes (*SACE_1284* and *SACE_5452*) were stably cloned and expressed in *E. coli* and found to encode β-glucosidase activity with genistin (unpublished results). If a strain of *S. erythraea* in which isoflavone glucoside conversion is blocked can eventually be constructed, it will most likely have to be engineered to have mutations in at least these two β-glucosidase genes as well as *eryBI*.

Our results also showed that *eryBI* knockout had no statistically significant effect on erythromycin production in either organism. This confirms previous reports that *eryBI* is not required for erythromycin production in *S. erythraea* (4, 18), and this conclusion is now extended to *A. erythreum*.

Finally, the primary purpose of this study was to study the effect of *eryBI* on isoflavone metabolism, and in this regard the results show that the *eryBI* β-glucosidase of *A. erythreum* can convert not only isoflavone glucosides but also flavone and flavonone glucosides to the corresponding aglycones. We also found that the metabolism of flavonoid compounds by *A. erythreum* *eryBI* stops after the formation of the aglycone. This means that *S. erythraea* and *A. erythreum* strains, in which the *eryBI* gene was functional, were constructed, it will most likely have to be engineered to have mutations in at least these two β-glucosidase genes as well as *eryBI*.

Unfortunately, the same cannot be said for the commercially important *S. erythraea* strain, in which the *eryBI* mutation does not result in a beta-glucosidase activity.
not stop the conversion of isoflavone glucosides. Furthermore, the metabolism of isoflavones does not stop with the formation of the aglycones in *S. erythraea*; the organism continues to create additional biotransformation products, based on the appearance of new spots that can be visualized by TLC (Fig. 4). We have begun an investigation of aglycone bioconversion and are continuing to further characterize the other components of the isoflavone glucoside bioconversion process in *S. erythraea*.

**ACKNOWLEDGMENTS**

This work was supported by Public Health Service grant CA093165 from the National Cancer Institute and by the U.S. Small Business Administration Office of Technology Small Business Innovation Research Program.

We acknowledge Ben Leach and Roy Wesley for helpful discussions.

**REFERENCES**