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Research

Characterization of the iron-regulated *desA* promoter of *Streptomyces pilosus* as a system for controlled gene expression in actinomycetes

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Abstract

Background: The bioavailability of iron is quite low since it is usually present as insoluble complexes. To solve the bioavailability problem microorganisms have developed highly efficient iron-scavenging systems based on the synthesis of siderophores that have high iron affinity. The systems of iron assimilation in microorganisms are strictly regulated to control the intracellular iron levels since at high concentrations iron is toxic for cells. *Streptomyces pilosus* synthesizes the siderofore desferrioxamine B. The first step in desferrioxamine biosynthesis is decarboxylation of L-lysine to form cadaverine, a desferrioxamine B precursor. This reaction is catalyzed by the lysine decarboxylase, an enzyme encoded by the *desA* gene that is repressed by iron.

Results: The binding of the DmdR (acronym for <u>d</u>ivalent <u>m</u>etal <u>d</u>ependent <u>r</u>epressor) to the *desA* promoter in presence of Fe^{2+} or other divalent ions has been characterized. A 51 bp DNA fragment of the *desA* promoter containing the 9 bp inverted repeat was sufficient for binding of the DmdR repressor, as observed by the electrophoretic mobility shift assay. The *desA* mobility shift was prevented by neutralizing DmdR with anti-DmdR antibodies or by chelating the divalent metal in the binding reaction with 2,2'-dipyridyl. Binding to the *desA* promoter was observed with purified DmdR repressors of *Streptomyces coelicolor* or *Rhodococcus fascians* suggesting that there is a common mechanism of iron-regulation in actinomycetes. The complete *desA* promoter region was coupled using transcriptional fusions to the *amy* reporter gene (encoding α-amylase) in low copy or multicopy *Streptomyces* vectors. The iron-regulated *desA* promoter was induced by addition of the iron chelating agent 2,2'-dipyridyl resulting in a strong expression of the reporter gene.

Conclusions: The iron-regulated *desA* promoter can be used for inducible expression of genes in *Streptomyces* species, as shown by de-repression of the promoter when coupled to a reporter gene.

Background

Iron is an important nutrient for microorganisms since it plays an essential role for cell growth. Iron acts as a cofactor of a large number of enzymes, forms part of cytochromes and is required for nitrogen fixation and DNA synthesis [1–3]. Despite its abundance in soil, the bioavailability of iron is quite low since it is usually present as insoluble complexes [3,4]. To solve the bioavailability problem microorganisms have developed highly efficient iron-scavenging systems based on the synthesis of
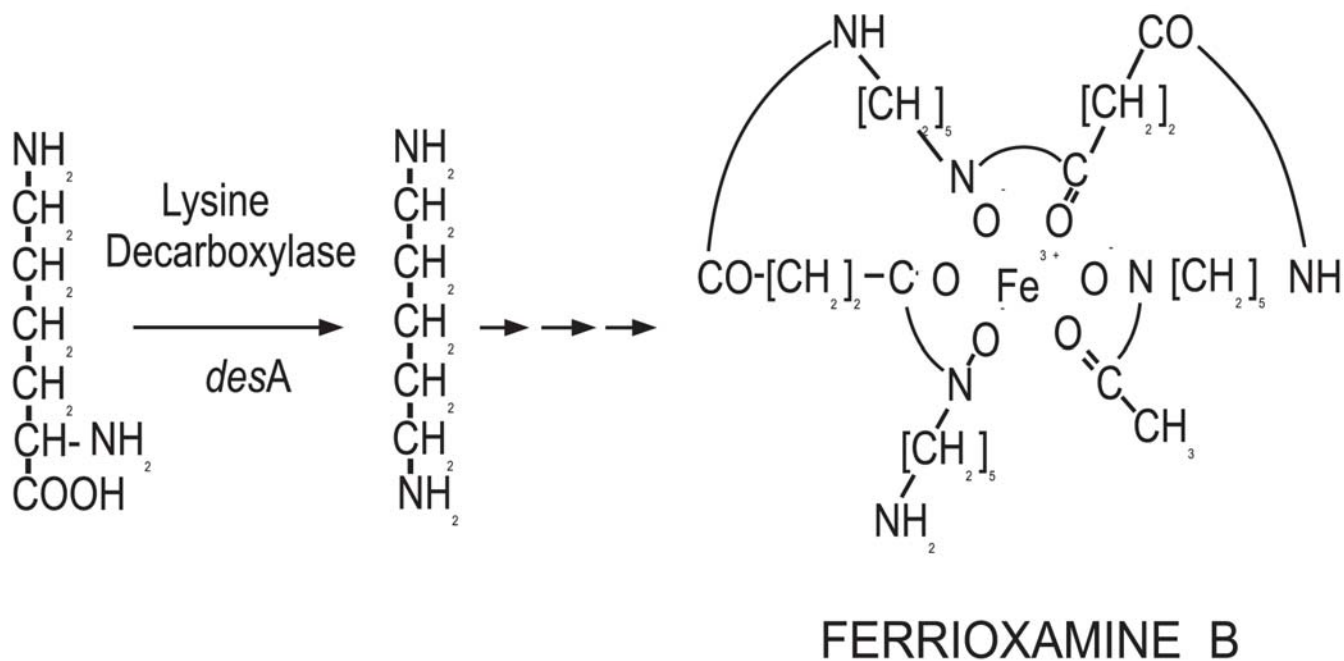
Figure 1
Biosynthesis of desferrioxamine B from lysine. The desferrioxamine B component binds Fe^{3+} to form ferrioxamine B. Lysine decarboxylase encoded by the *desA* gene catalyzes the first step of the biosynthetic pathway.

siderophores that have high iron affinity [5–7]. The systems of iron assimilation in microorganisms are strictly regulated to control the intracellular iron levels since at high concentrations iron is toxic for cells [8–15]. Siderophores form a six-coordinated octaedral complex with ferric iron with extremely high affinity (stability constants) ranging from 10^{23} to 10^{52}.

Streptomyces pilosus synthesizes the siderofore desferrioxamine B [16]. Desferrioxamine B is used clinically to treat disorders related to iron overload and pathological iron deposition in man [17]. The first step in desferrioxamine biosynthesis is decarboxylation of L-lysine to form cadaverine, a desferrioxamine B precursor (Fig. 1). This reaction is catalyzed by the lysine decarboxylase, an enzyme encoded by the *desA* gene that is repressed by iron [18,19].

The *desA* promoter possess a region involved in iron regulation containing a symmetrical 19 bp sequence that overlaps with the -10 box and the transcription initiation site of the *desA* gene [20]. This palindromic sequence is very similar to the iron box recognized by the DtxR iron regulator of *Corynebacterium diphtheriae* [21] and the *dmdR* gene of *Corynebacterium* (formerly *Brevibacterium*) *lactofermentum* [22]. A DmdR protein similar to DtxR occurs in

several *Streptomyces* strains [23] (Flores FJ and Martín JF, unpublished results).

The availability of iron-regulated promoters may provide a very useful biological sensor to test the presence of iron in different environments. In addition, an iron-regulated promoter provides an interesting tool for controlled expression of genes at specific times during the fermentation by addition of either iron or iron-chelating agents.

It was, therefore, of great interest to characterize the binding of the DmdR repressor to the *desA* promoter and to study the derepression of the *desA* gene of *S. pilosus* following addition of iron chelators.

We report in this article that DmdR repressors from different actinomycetes bind to the *desA* promoter in presence of iron making this system an excellent regulated promoter for inducible or repressible expression of genes in different actinomycetes.

Results
Characterization of the **desA** *promoter*
A detailed analysis of the promoter region of the *desA* gene of *S. pilosus* was performed to define the regulatory region involved in iron-regulation and to study if the *desA* pro-
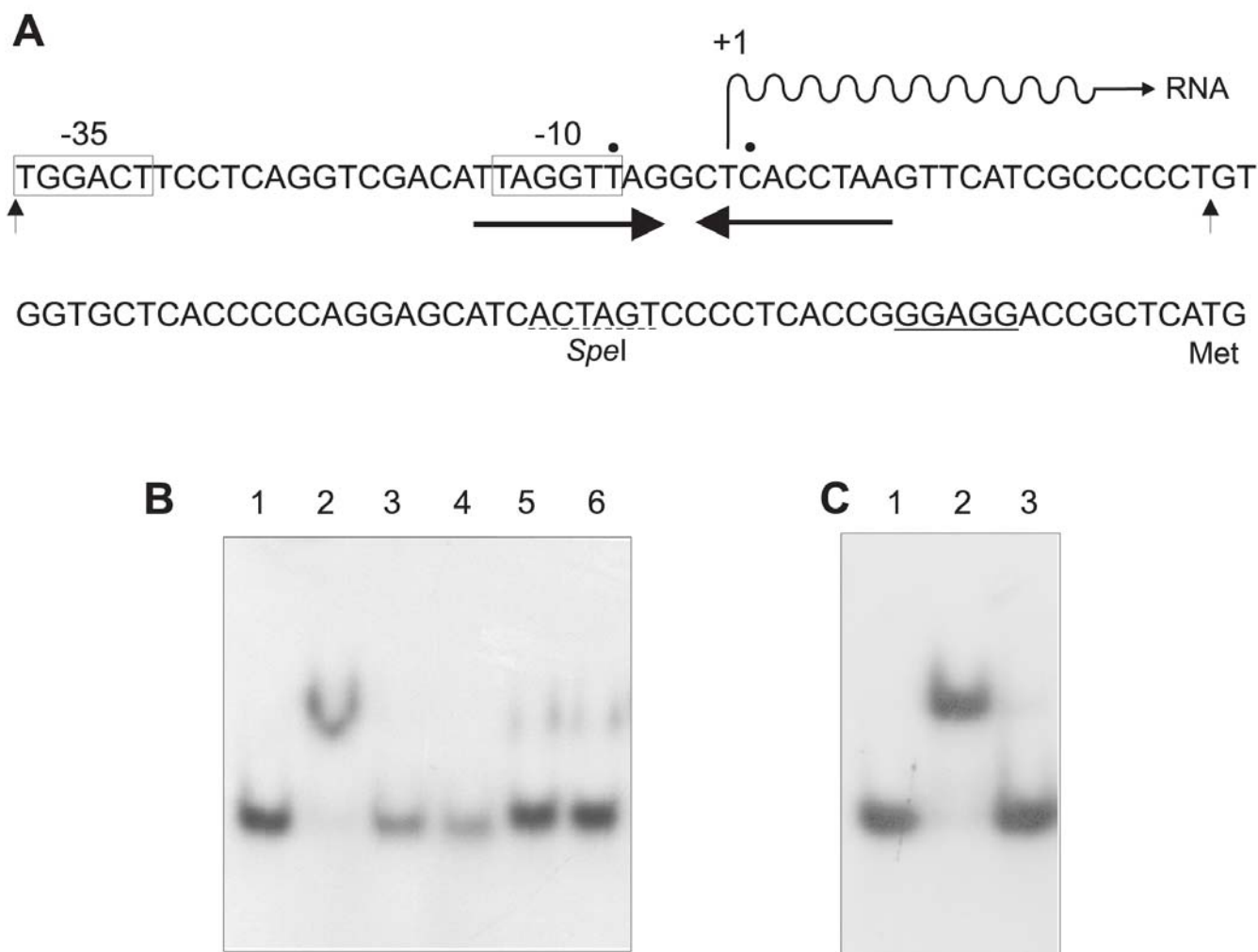
Figure 2
A Nucleotide sequence of the promoter operator region of the *desA* gene containing the -10 and -35 boxes; the transcription start point is indicated as +1. The mRNA is indicated by a wavy line and the putative ribosome binding site GGAGG upstream of the first translated codon ATG is underlined. The convergent thick arrows indicate the palindromic sequence of binding of the DmdR protein (operator). The two vertical arrows indicate the DNA fragment used for the gel mobility shift experiments.
B Electrophoretic mobility shift assay of the [^{32}P]labeled *desA* probe and DmdR protein from *S. coelicolor*. Lanes 1, labeled free *desA* probe; 2, labeled *desA*, mixed with DmdR (note the band shift); 3, labeled *desA* with excess unlabeled *desA*; 4, labeled *desA* with DmdR and anti-DmdR antibodies; 5, labeled *desA* with DmdR and 2,2'-dipyridyl (200 mM) without Mn^{2+}; 6, labeled *desA* with DmdR and 2,2'-dipyridyl (200 mM). **C** Same as in B but with DmdR purified from *R. fascians*. Lanes 1, labeled *desA*; 2, labeled *desA* with DmdR; 3, labeled *desA* with DmdR and excess unlabeled *desA*.

moter could be regulated by the DmdR repressor of different actinomycetes. As shown in Fig. 2, the *desA* promoter region contains a 19 bp palindromic region overlapping with the transcription initiation site defined by Günter et al. [20]. To ascertain that this putative repressor binding sequence was involved in iron regulation a DNA fragment of 51 bp extending from -31 to +21 was synthesized, labeled and used as a probe in electrophoretic mobility shift assays. DmdR proteins purified from *Streptomyces coeli-*

color and *Rhodococcus fascians* were used for the binding assays in the electrophoretic mobility shift assays.

As shown in Fig. 2B and 2C, a strong shift of the *desA* promoter was observed in presence of the pure DmdR protein of either *R. fascians* or *S. coelicolor*. The mobility shift was removed by neutralizing the DmdR protein with anti-DmdR antibodies (Fig. 2B, lane 4) or by adding unlabeled excess *desA* probe (lane 3). The mobility shift required a

divalent metal and was prevented by addition of the chelator 2,2-dipyridyl.

Constructions with the desA promoter in monocopy and multicopy vectors

The promoterless *amy* gene of *S. griseus* IMRU 3570 was subcloned as a 1.8 kb *Eco*RI-*Eco*RV band from plasmid pAM2PP [24] into the vector pTC191 (digested with *Sma*I-*Eco*RI) giving rise to plasmid pTCA. The promoter of the *S. pilosus desA* gene was subcloned from plasmid pTQ217 (a derivative of pTQ209) [19] as a 0.8 kb *Ecl*136II-*Spe*I DNA fragment upstream of the promoterless *amy* gene, giving rise to plasmid pTCDA. The correct orientation was confirmed by restriction endonuclease mapping of the construction. The construction with the incorrect orientation of the *desA* promoter with respect to the *amy* reporter gene was named pTCADi and was used as a negative control.

Both, the direct and the inverted *desA-amy* fusions, were subcloned as *Hin*dIII fragments into the multicopy *Streptomyces coelicolor* vector pIJ699 [25] and the low-copy pIJ2842 vector [26]. The constructions derived from the multicopy plasmid were named pUL99DA and pUL99ADi, respectively, whereas the constructions derived from the low-copy vector were designated as pUL42DA and pUL42ADi, respectively (Fig. 3).

Transformants with the pUL99DA and pUL42DA showed a strong expression of the amylase reporter gene when tested directly on colonies growing on plates of starch-containing LS medium with 2,2'-dipyridyl. Transformants with plasmids pUL99ADi and pUL42ADi that contained the *desA* promoter coupled in the inverted (incorrect) position to the *amy* gene did not show any expression of the *amy* gene and produced only the background halo of starch digestion due to the endogenous α-amylase of the *S. coelicolor* host (Fig. 4).

Expression of the desA-amy fusion is regulated by iron in multicopy and low-copy plasmids

To test the effect of iron on expression of the *desA-amy* fusion, the formation of amylase was quantified in *S. coelicolor* transformants containing plasmids pUL99DA or pUL42DA. *S. coelicolor* cells were grown in YEME + 34% sucrose collected and suspended in LS medium as indicated in Materials and Methods. To quantify the response to iron-depletion, iron in this medium was removed by addition of the chelating agent 2,2'-dipyridyl. Results showed that there was a seven-fold increase in the synthesis of the reporter enzyme in cells deprived of iron (Fig. 5A). When iron was complexed with the chelating agent, a large increase in the reporter enzyme activity was observed until 60 h of incubation while in cells growing in iron the amylase activity declined after 36 h.

Similar results were also observed when the reporter α-amylase activity was expressed as specific activity per μg of DNA (dry weight can not be determined in starch-containing media). Although total amylase accumulated until the 60 h the highest specific rate of *desA* expression as measured by the specific rate of α-amylase peaked at 12 h following addition of the iron chelator (Fig. 5B).

Constructions containing the *desA* promoter in multicopy plasmids might, however, titrate the DmdR repressor. It was, therefore, of interest to test the effect of iron on low-copy plasmids (1 or 2 copies per genome).

Constructions carrying the *desA* promoter in the low-copy plasmid pUL42DA were also strongly regulated. Removal of iron with 2,2'-dipyridyl produced a two-fold increase in the expression of the reporter gene (Fig. 5B). The same effect was also observed when the reporter activity was expressed as specific amylase activity (Fig. 5C). In low-copy number transformants the reporter amylase activity decreased after 36 hours whereas in the high copy number transformants it accumulated in the culture until 60 h, reflecting a much higher ability to produce α-amylase in the transformants with the multicopy plasmids.

Taken together these results indicate that the *desA* promoter is regulated in the multicopy pIJ99DA transformant as well as in low-copy transformants. The DmdR repressor level is sufficient to keep most of the *desA* copies repressed under iron excess conditions even in transformants containing a high number of copies (about 50 copies per cell).

Iron-regulated versus non-regulated promoters as tools for gene expression

The iron-regulated *desA* promoter serves as an inducible system for expression of genes following iron removal with 2,2'-dipyridyl. Previously, we compared the strength of different *Streptomyces* promoters by coupling them to the *amy* reporter gene [27]. The best *Streptomyces* promoter available in our hands is the *saf* gene promoter that drives expression of the secretion activating factor (*saf*). The transcription initiation ability of the *desA* promoter was compared with that of *saf* promoter in plasmids containing the same replicon (from pIJ101).

Results of the comparative study (Fig. 6) showed that whereas the *desA*-mediated expression of the reporter gene was strongly regulated by iron, expression from the *saf* promoter was largely insensitive to iron starvation.

The levels of amylase were about 50% higher in cultures expressing the reporter gene from the *desA* promoter under derepression conditions than in transformants
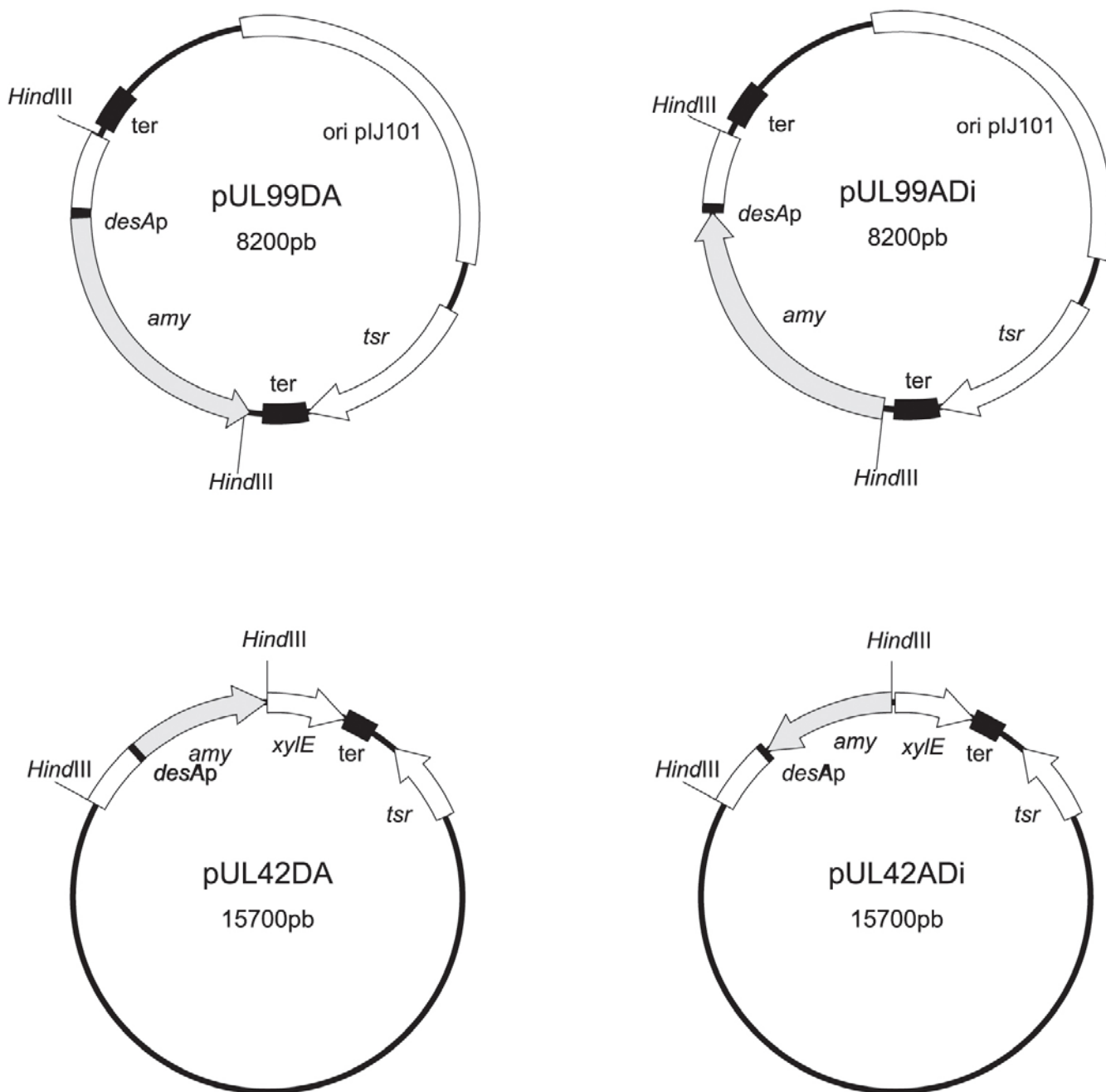
Figure 3
Physical map of the four vectors used to quantify iron regulation of the *desA* promoter. pUL99DA and pUL99ADi are multi-copy vectors whereas pUL42DA and pUL42Adi are low copy number vectors; *amy* indicates the promoter-less *amy* gene; *desAp* corresponds to the *desA* promoter; *xylE*, catechol oxygenase gene; *ter*, transcriptional terminator; *tsr*, thiostrepton resistance gene (see Table 1).
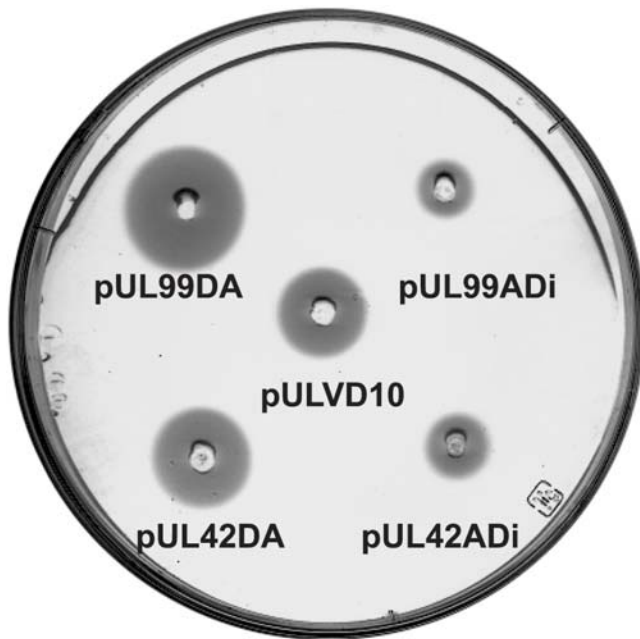
Figure 4
Direct test on agar plates of the reporter α-amylase expression ability of the *desA* promoter using round patches of growth of pUL99DA and pUL42DA transformants in LS medium containing 2,2'-dipyridyl as compared to transformants with pULVD10 in which the reporter amylase gene is under the control of the *saf* promoter. Control transformants with pUL99ADi and pUL42ADi are shown on the right. The plate was stained with I_2 + IK; the figure is a negative of the plate photograph.

expressing the reporter from the *saf* promoter (either under iron repressing or derepressing conditions).

Discussion

In bacteria and lower eurcaryotes iron is scavenged and transported into the cells by high-affinity iron-chelators (siderophores). When the concentration of iron in the environment is low, transcription of the siderophore-encoding genes is triggered. The specific transcriptional response of the genes encoding the siderophores and their receptors is an attractive subject because of its biotechnological interest [5].

In recent years considerable advances were made in the characterization of the DtxR repressor of *C. diphtheriae* that mediates iron-regulation. The regulatory protein DtxR binds to a 9 bp-interrupted palyndrome in the diphtheria toxin gene (see review by Tao et al. 1994). We have found previously that genes similar to *dtxR* that are generically named *dmdR* (for divalent metal dependent regulator) occur in *Corynebacterium* (formerly *Brevibacteri-*
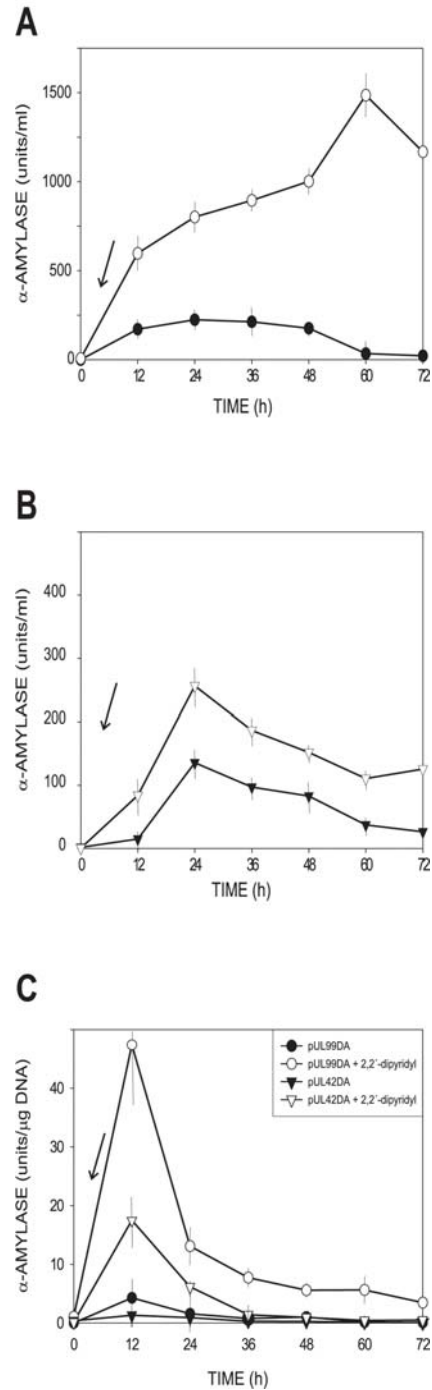

Figure 5
Iron regulation of expression of the reporter α-amylase gene coupled to the *desA* promoter in a multicopy transformant (pUL99DA) (panel A) and a low-copy number transformant (pUL99DA) (panel B) expressed as volumetric enzyme activity (panels A and B) or as specific activity (panel C). ○, ●, pUL99DA; ▼, ▽, pUL42DA. Closed symbols, control cultures in LS medium; open symbols, cultures induced with the iron-complexing agent 2,2'-dypirydyl added at time 0 (inclined arrows).
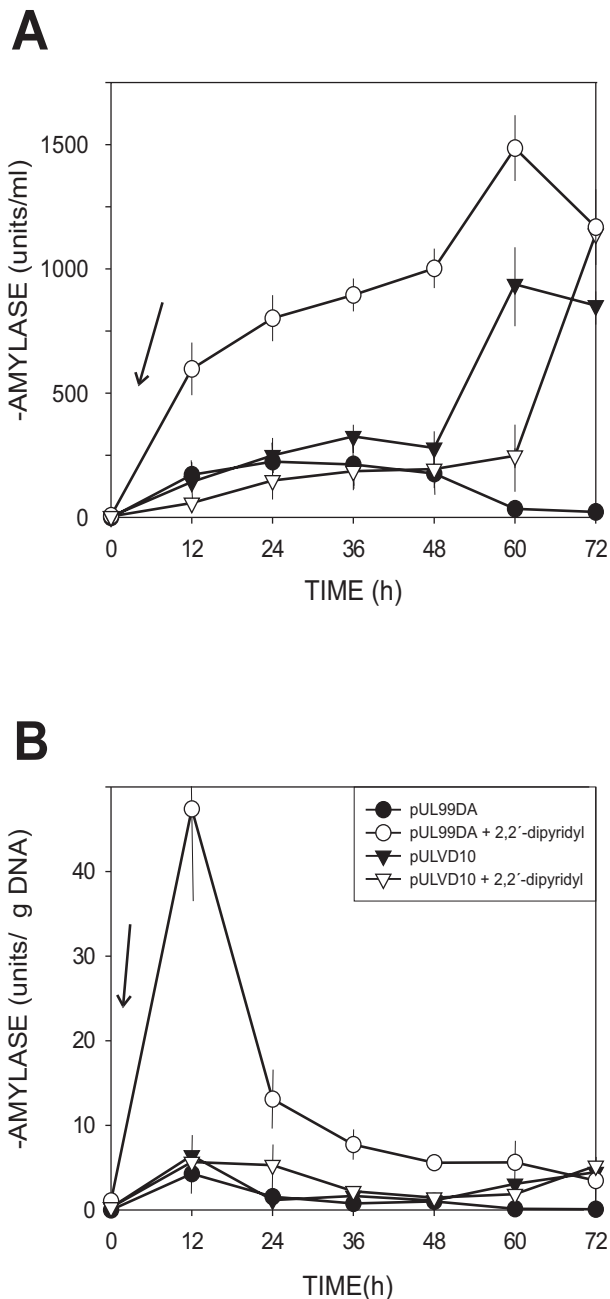
A

B

Figure 6
Expression of the reporter α-amylase gene from the iron-regulated *desA* promoter versus the non-regulated *saf* promoter. Experimental conditions were as in Fig. 5. ○, ●, pUL99DA; ▼, ▽, pULVD10. Closed symbols control cultures in LS medium; open symbols cultures induced by addition of the iron-chelator 2,2'-dipyridyl at time 0 (vertical arrows). Results are given as volumetric enzyme levels (panel A) or as specific enzyme production (panel B). The inclined arrows indicate the time of addition (0 h) of the 2,2'-dipyridyl inducer

um) lactofermentum [22] in *Rhodococcus fascians* (J. Rincon and J.F. Martín, unpublished) and several *Streptomyces* species (Flores FJ and Martín JF, unpublished).

A few DmdR responsive operators occurring in the promoter regions of iron-regulated genes have been analyzed. Most of the information available refers to the *tox* gene of *C. diphtheriae*. The promoter of the *desA* gene of *Streptomyces pilosus* contains one of the consensus DmdR-binding sequences [20]. As shown in this article, purified DmdR repressor proteins from two different actinomycetes are able to interact with the 51 bp region containing the *desA* operator. The interaction required a divalent metal ion, either Fe^{2+}, Mn^{2+} or Co^{2+}. All the available evidence indicates that there is a common mechanism of iron regulation mediated by repressors of the DmdR family in corynebacteria and in higher actinomycetes.

There is a need of characterizing well-defined regulated promoters as tools for controlled gene expression in actinomycetes. Günter and coworkers [20] showed that the regulation of desferrioxamine B production is exerted at the transcriptional level. The concentration of iron in culture media can be regulated very simply by addition of the 2,2'-dipyridyl chelator [22] and, therefore, as shown in this article, expression from the *desA* promoter can be triggered at will by iron starvation at different times. There is no endogenous *desA* gene in most actinomycetes since this gene appears to be specific for secondary metabolism [19].

When the reporter activity was determined, we observed regulation both in low-copy number expression vectors and in multicopy vectors. It seems, therefore, that enough molecules of the *DmdR* iron regulator exist in *S. coelicolor* to bind the copies of the *desA* promoter in the multicopy vector. DNA binding to the *desA* promoter as shown by electrophoretic mobility shift assay occurred with the *DmdR* repressors of *S. coelicolor*, *R. fascians* (F. Flores and J.F. Martín, unpublished) and *S. lividans* [23]. The presence in several actinomycetes of a DmdR repressor (F.J. Flores and J.F. Martín, unpublished) makes this iron-regulated expression system very attractive for tailored expression of homologous or heterologous genes in a variety of actinomycetes.

Conclusions

- The iron-box of the iron-regulated *desA* promoter of *S. pilosus* has been characterized by the electrophoretic mobility shift assay (EMSA) technique using the purified DmdR repressor proteins of *S. coelicolor* and *R. fascians* that interact with the iron-box

Table 1: Plasmids used in this work

Plasmid	Description	Reference
pTC191	A derivative of pUC19 containing identical restriction sites on both sides of the polylinker	[36]
pAM2PP	Contains the promoter-less *amy* gene (encoding the *S. griseus* α-amylase)	[24]
pTQ217	A derivative of pTQ207 (Günter et al. 1993) containing the *desA* gene of *S. pilosus* encoding the enzyme lysine decarboxylase	T. Schupp, personal communication
pIJ699	Bifunctional vector *E. coli-Streptomyces* of high copy number. The functional region in *Streptomyces* is flanked by transcriptional terminators. Allows positive selection of transformants	[25]
pULVD10	A derivative of pIJ699 that contains the promoter-less *amy* gene expressed from the *saf* promoter (*saf*p) of *S. griseus*	[27]
pIJ2842	Low-copy number promoter-probe vector for *Streptomyces* promoters	[26]
pTCA	A derivative of pTC191 containing the *amy* gene without its promoter in a 1.8 kb DNA fragment	This work
pTCDA	A derivative of pTCD containing the *desA* promoter (*desA*p) coupled to the 5' end of the promoter-less *amy* gene	This work
pTCADi	A derivative of pTCD containing the *desA* promoter fused to the 3' end of the *amy* gene (negative control)	This work
pUL99DA	A derivative of the multicopy pIJ699 containing the transcriptional fusion *desA*p-*amy* in the correct orientation	This work
pUL99ADI	A derivative of pIJ699 containing the incorrect transcriptional fusion *amy*-*desA*p (non-functional)	This work
pIJ42DA	A derivative of the low-copy pIJ2842 containing the transcriptional fusion *desA*p-*amy* in the correct orientation	This work
pIJ42ADI	A derivative of the low-copy pIJ2842 containing the incorrect transcriptional fusion *amy*-*desA*p no functional	This work

- The iron-regulated *desA* promoters has been coupled to the reporter *amy* gene (encoding α-amylase) in low copy and high copy *Streptomyces* vectors.

- The *desA* promoter is strongly induced by addition of the iron-chelating agent 2,2'-dipyridyl.

- This iron-regulated *desA* promoter can be used for controlled expression of other transcriptionally-coupled genes in *Streptomyces* species. Expression from this promoter is switched off by iron and switched-on by 2,2'-dipyridyl.

Methods
Microorganisms, plasmids and culture conditions
Streptomyces coelicolor A3(2) was routinely used as a model actinomycete. S. coelicolor cultures were grown in YEME (yeast extract 10 g/l, malt extract 10 g/l) with 34% sucrose to obtain disperse growth [28] or in defined Lechevalier medium [29] supplemented with 10 g/l soluble starch (LS medium) for quantification of amylase production. Thiostrepton was added to the culture medium to maintain the plasmids in the transformants (final concentration 25 mg/ml in solid medium; 5 mg/ml in liquid media). All plasmids used in this work are listed in Table 1. E. coli DH5α was grown in TB medium [30] or in LA medium as indicated by Hannahan [31]. E. coli transform-

ants were selected on LA plates containing ampicillin (100 μg/ml). 2,2'-dipyridyl was added, when required, at a final concentration of 250 μM.

DNA procedures
Transformation conditions for *S. coelicolor* and plasmid DNA isolation from the transformants were as described by Hopwood et al. [32]. DNA digestion with restriction endonucleases, end-filling and removal of 5' or 3'-protruding ends were performed by standard procedures [33].

Preparation and labeling of the DNA probes
A 51 bp DNA fragment containing the 19 bp inverted repeat based on the nucleotide sequence of the *desA* gene [20] was synthesized by annealling two 47 bp oligonucleotides (with stagered ends) and subcloning the 51 bp DNA fragment in pBluescript KS+. The insert was sequenced to confirm the nucleotide sequence. After plasmid amplification the probe was scissed from the vector and isolated from the agarose gel by the Quiaex procedure (Quiagen) and labeled by end-filling with the Klenow fragment of the DNA polymerase and $[^{32}P]\alpha$-dCTP.

Electrophoretic mobility shift assays
The electrophoretic mobility shift assay was performed as described by Tao et al. [34] using as probes DNA frag-

ments containing the *desA* regulatory region labeled by end-filling with Klenow DNA polymerase. The His-tagged DmdR protein of *R. fascians* was purified by filtration through a Ni $^{2+}$-NTA column after cloning the *R. fascians dmdR* in the expression vector pQE (Quiagen). The DmdR1 protein of. *S. coelicolor* was purified as a GST-fused protein by filtration through a Glutathion-Sepharose 4B affinity column (Amersham-Pharmacia Biotech). The *dmdR1* gene was first cloned in a pGEX expression vector (Amersham-Pharmacia). Following elution of the GST-DmdR1 fused protein from the column, the DmdR1 protein was released by cleavage with thrombin as indicated by the manufacturer. The DmdR binding reaction was performed for 30 min at 30°C in a final volume of 30 µl containing 5 mM MgCl$_2$, 40 mM KCl, 2 mM DTT, 125 mM MnCl$_2$, 10% (v/v) glycerol, 1 µg of poli dI-dC, 8.5 µg BSA, purified DmdR protein (about 20 ng) and the *desA* labeled probe.

Quantification of the reporter α-amylase activity
The α-amylase activity was determined by quantifying the maltose released from soluble starch with 3,5-dinitrosalycilic acid [35]. All cultures in LS medium were inoculated with 1 ml of cells (O.D. = 1) grown in YEME + 34% sucrose washed twice with sterile saline solution. One unit of amylase was defined as the activity that forms one nmol of maltose per minute. The specific activity is given as units per µg of DNA in the cells.

Abbreviations
BSA, bovine seroalbumin; LA, Luria agar; TB, triptic broth; DTT, dithiothreitol; LS, Lechevalier starch.

Authors' contributions
JFM directed the research and designed the experimental work. FJF performed most of the experimental work in the article. JR helped with the EMSA assays. All authors read and approved the final manuscript.

Acknowledgements

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