Relaxed \textit{rrn} expression and amino acid requirement of a \textit{Corynebacterium glutamicum rel} mutant defective in (p)ppGpp metabolism

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Abstract

The stringent response in \textit{Corynebacterium glutamicum} was investigated. Sets of \textit{rrn}–\textit{cat} fusions were constructed in their native chromosomal position to examine the effects of amino acid starvation in a rel\textsuperscript{+} strain and a \textit{Δrel} mutant defective in (p)ppGpp metabolism. The expression of the six \textit{rrn} operons in the rel\textsuperscript{+} control was stringently regulated and reduced to 79\% upon induction of amino acid starvation. The \textit{Δrel} mutant displayed a relaxed regulation and was unable to reduce the \textit{rrn} expression under amino acid depletion conditions. In addition, the \textit{Δrel} mutant grew more slowly in minimal medium than a rel\textsuperscript{+} control. This growth effect was restored by a plasmid-encoded copy of rel or, alternatively, by supplementation of the minimal medium with the amino acid mixture casamino acids. In particular, the \textit{Δrel} strain of \textit{C. glutamicum} displayed a requirement for the amino acids histidine and serine. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Stringent response; \textit{rrn} expression; \textit{Corynebacterium}

1. Introduction

The stringent response is a pleiotropic physiological response leading to rapid adaptation of cell growth to nutrient depletion (reviewed in [1]). Many features of the stringent response are believed to be mediated by the hyperphosphorylated guanosine nucleotides ppGpp (guanosine 5\'-diphosphate 3\'-diphosphate) and pppGpp (guanosine 5\'-triphosphate 3\'-diphosphate), collectively abbreviated (p)ppGpp. In \textit{Escherichia coli}, (p)ppGpp is synthesized predominantly by the ribosome-bound RelA protein which is activated when uncharged tRNA binds to the acceptor site of translating ribosomes. Therefore, the synthesis of (p)ppGpp can be experimentally invoked by amino acid depletion or, alternatively, by addition of tRNA synthetase inhibitors, such as serine hydroxamate. The SpoT protein is a homologue of RelA and responsible for (p)ppGpp degradation in \textit{E. coli}. The residual level of (p)ppGpp synthesis activity observed in a rel\textsuperscript{A} mutant strain most probably reflects a synthetic role of SpoT in addition to its degradative function [2]. The RelA protein senses the tRNA aminoacylation ratio and indirectly reduces the protein biosynthesis by repression of stable RNA synthesis [3]. Cells that are unable to repress stable RNA synthesis under amino acid depletion conditions have been termed relaxed. Accordingly, a rel\textsuperscript{A} mutant of a marine \textit{Vibrio} strain failed to accumulate (p)ppGpp in the presence of serine hydroxamate and displayed a relaxed control of RNA synthesis [4]. In addition, \textit{E. coli} strains apparently lacking (p)ppGpp showed a complex phenotype including auxotrophy for several amino acids and morphological alterations [2].

Numerous experimental findings in \textit{Streptococcus equisimilis} [5] and \textit{Bacillus subtilis} [3] as well as whole-genome sequences of other Gram-positive bacteria such as \textit{Mycobacterium tuberculosis} suggest that the stringent response of these micro-organisms is mediated by a single \textit{relAlspoT} homologue encoding a bifunctional protein capable of
both (pppGpp synthesis and degradation. In the Gram-positive soil bacterium *Corynebacterium glutamicum*, which is widely used for the fermentative production of amino acids, a relA/spoT homologous gene, termed rel, has been cloned, sequenced and deleted [6]. The rel gene was shown to encode a bifunctional enzyme with (pppGpp synthetase and (pppGpp degrading activities. A defined deletion mutant failed to accumulate (pppGpp upon addition of serine hydroxamate [6].

In this paper we studied the expression of the *rrn* operons of *C. glutamicum* to understand the stringent response and the mechanisms triggering amino acid biosynthesis in this micro-organism in more detail. We could assess the relaxed *rrn* expression of a *C. glutamicum* rel mutant by means of chromosomal *rrn-cat* fusions. In addition, growth analyses in minimal medium revealed that (pppGpp may play a role in the biosynthesis of histidine and serine.

### 2. Materials and methods

#### 2.1. Media, growth conditions and standard genetic techniques

*C. glutamicum* and *E. coli* strains were routinely grown in LBG medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 2 g glucose per liter; pH 7.4) at 30 and 37°C, respectively. The growth of *C. glutamicum* was monitored in mineral medium [7] supplemented with 0.1% casamino acids (Difco Laboratories, Detroit, MI, USA) or an incomplete set of amino acids (20 aa minus X, all at 50 μg ml⁻¹) by means of the spectrophotometer Novaspec II (Pharmacia, Freiburg, Germany) at 580 nm. Kanamycin was used for selection in *E. coli* (50 μg ml⁻¹) and *C. glutamicum* (25 μg ml⁻¹). Plasmid DNA of *E. coli* was extracted by means of the QIAprep spin kit (Qiagen, Hilden, Germany). Restriction fragments were purified from 0.8% agarose gels with the QIAEX II gel extraction kit (Qiagen). Recombinant plasmids were introduced into *E. coli* DH5αMCR by electroporation.

#### 2.2. Construction of *rrn-cat* fusions in the chromosome of *C. glutamicum*

Transposon Tn1736Km contains the promoterless Tn9-derived cat gene on a 780-bp BamHI gene cartridge [8]. The cat-carrying DNA fragment was purified from an agarose gel and cloned into the BamHI site of pK19mob [9]. The resulting vector was cleaved with HindIII and SphI and ligated with a 1.5-kb HindIII–SphI fragment (Fig. 1A) purified from prR14 which carries a chromosomal PstI fragment with *rrnE* from *C. glutamicum* ATCC 13032 [10]. The resulting vector, termed pK19mob16Scat (Fig. 1B), was transferred to *C. glutamicum* RES167 (University of Bielefeld) and *C. glutamicum* RES167Δrel [6] by conjugation. Mating experiments were performed according to the protocol described by Schäfer et al. [11]. Integration of the plasmid was verified by Southern hybridization. For this purpose, chromosomal DNA was prepared as described previously [12], digested with PstI and separated on 0.8% agarose gels. Southern hybridization was performed on Hybond-N nylon membranes (Amerham, Braunschweig, Germany) using the vacuum blotter VacuGene (Pharmacia). Fixation of DNA, labeling of DNA probes and hybridization were performed with the DIG DNA Labeling and Detection kit from Boehringer (Mannheim, Germany). The identified strains carrying pK19mob16Scat integrated into the *rrn* operons were named *C. glutamicum* RES167 *rrnA-cat* to *rrnF-cat* and *C. glutamicum* RES167Δrel *rrnA-cat* to *rrnF-cat*, respectively. The nucleotide sequence of the *rrnE* operon from *C. glutamicum* ATCC 13032 has been deposited in the GenBank database under accession number AF314192.

#### 2.3. Cat (chloramphenicol acetyltransferase) enzyme-linked immunosorbent assay (ELISA) assay

Whole-cell extracts were prepared from *C. glutamicum* cells grown to a cell density of 4.5×10⁶ ml⁻¹ in mineral medium [7] supplemented with 50 μg ml⁻¹ kanamycin. Subsequently, 10-ml portions of cells were harvested by centrifugation at 5500×g and washed twice with distilled H₂O. The cell pellet was resuspended in 0.7 ml Tris–HCl (250 mM, pH 7.0) and lysed in a RiboLyser BLUE tube (Hybaid Ltd., Teddington, UK). Disruption of *C. glutamicum* cells was performed by means of the RiboLyser instrument at a speed rating of 6 for two time intervals of 30 s. The lysates were cleared by centrifugation for 40 min at 5500×g and 4°C. The total protein content of the crude extract was determined with the Bio–Rad Protein Assay (Bio–Rad Laboratories, Munich, Germany). The Cat protein determination was performed according to the description of the Cat ELISA assay from Boehringer (Mannheim, Germany). The fluorescence was measured with 4-methylumbelliferyl phosphate as substrate (Sigma, Deisenhofen, Germany) and the Fluoromark microplate fluorometer from Bio–Rad Laboratories (excitation: 355 nm; emission: 460 nm).

### 3. Results and discussion

#### 3.1. Relaxed *rrn* expression in a rel mutant of *C. glutamicum* upon amino acid starvation

During the stringent response complex changes in the pattern of gene expression occur, including an immediate reduction of ribosomal RNA (*rrn*) operon transcription [1,13]. To investigate this aspect in *C. glutamicum*, we have compared the expression of the six *rrn* operons, *rrnA* to *rrnF*, in their native chromosomal location by
means of \textit{rrn\textsuperscript{-}cat} fusions. For this purpose, a 1.5-kb \textit{Hind}\textsuperscript{III}\textendash \textit{Sph}\textsuperscript{I} fragment of the \textit{rrnE} region was fused to a promoterless \textit{cat} gene (Fig. 1A). Subsequently, the plasmid construct was integrated into the chromosome of \textit{C. glutamicum} RES167 and \textit{C. glutamicum} RES167\textsuperscript{rel} by homologous recombination. Since the nucleotide sequence of the \textit{Hind}\textsuperscript{III}\textendash \textit{Sph}\textsuperscript{I} fragment should be virtually identical in all \textit{rrn} operons, each of the chromosomal copies could be disrupted by the single vector construct pK19mob16S\textit{cat} (Fig. 1A) and fused to the promoterless \textit{cat} gene. The integration of pK19mob16S\textit{cat} into the six \textit{rrn} operons was analyzed by Southern hybridization. Chromosomal DNA was isolated, digested with \textit{Pst}\textsuperscript{I} and probed with labeled pK19mob16S\textit{cat} DNA. Since each of the six \textit{rrn} operons is located on a chromosomal \textit{Pst}\textsuperscript{I} fragment of unique size (Fig. 1B), the native \textit{Pst}\textsuperscript{I} band characteristic of each operon should be absent from the blot upon vector integration. Fig. 1B presents a Southern blot of chromosomal digests showing each of the constructed fusion strains of \textit{C. glutamicum} RES167\textsuperscript{rel}. Since the integration of pK19mob16S\textit{cat} into all \textit{rrn} operons of \textit{C. glutamicum} resulted in gene disruption, this experiment indicated that none of the six operons was essential under the conditions used for selection.

![Fig. 1. Construction of \textit{rrn\textsuperscript{-}cat} fusions in the chromosome of \textit{C. glutamicum}. (A) A restriction map of the \textit{Pst}\textsuperscript{I} fragment carrying \textit{rrnE} of \textit{C. glutamicum} is shown. The gene organization was deduced from GenBank entry AF314192. The mobilizable (\textit{mob}) vector pK19mob16S\textit{cat} carries the \textit{Hind}\textsuperscript{III}\textendash \textit{Sph}\textsuperscript{I} DNA fragment of \textit{rrnE} fused to the promoterless \textit{cat} gene. (B) Southern analysis of RES167\textsuperscript{rel} strains with the various chromosomal \textit{rrn\textsuperscript{-}cat} fusions. A \textit{Pst}\textsuperscript{I} digest of chromosomal DNA was probed with digoxigenin-labeled pK19mob16S\textit{cat}. Strains carrying the fusion construct lack the characteristic band of the wild-type operon. Due to the presence of a \textit{Pst}\textsuperscript{I} site in pK19mob16S\textit{cat}, the band is replaced with two signals of defined size. RES167 was used as control strain (R). Letters above the lanes indicate the particular \textit{rrn\textsuperscript{-}cat} fusion strain. A digoxigenin-labeled DNA size marker (M) was used. Identical Southern data were obtained with RES167 as recipient strain (data not shown).](image1)

![Fig. 2. Cat activities of the chromosomal \textit{rrn\textsuperscript{-}cat} fusions in \textit{C. glutamicum}. (A) Chromosomal fusions of \textit{C. glutamicum} RES167\textsuperscript{rel} (black) and the \textit{rel\textsuperscript{rel}} control \textit{C. glutamicum} RES167 (white) were assayed in mineral medium. (B) Effect of amino acid starvation on Cat activity. Amino acid limitation was induced by the addition of 1 mg ml\textsuperscript{-1} serine hydroxamate to exponentially growing cultures in mineral medium (\textit{OD}\textsubscript{580 nm} = 0.45). The amount of Cat protein was monitored 45 min after addition of the analogue. Results are the average of three independent assays.](image2)
Subsequently, the two sets of RES167 and RES167Δrel strains carrying the rrn–cat fusion were assayed for the presence of Cat by an ELISA technique. All of the operons were expressed during exponential growth in LBG medium (Fig. 2A). The level of cat expression varied over a 1.5-fold range. The highest expression was measured for the rrnA– and rrnF–cat fusions. No significant difference was observed between RES167Δrel and the rel mutants control RES167 (Fig. 2A). The six operons rrnA to rrnF are located in non-contiguous sites around the chromosome with rrnA and rrnF being located close to the origin of replication of C. glutamicum [10]. This location should result in a higher copy number of the respective genes during growth and the gene dosage should contribute to the amount of Cat protein determined in fusions in their native chromosomal context.

In addition, the rrn–cat fusions were examined in response to amino acid starvation induced by serine hydroxamate, a structural analogue of l-serine (Fig. 2B). Addition of serine hydroxamate to C. glutamicum cultures resulted in a rapid accumulation of (ppp)Gpp, although with apparently lower (ppp)Gpp quantities than observed in E. coli [6,14]. After addition of the analogue to exponentially growing cultures (optical density at 580 nm (OD580 nm) = 0.45), the bacteria continued to grow exponentially but with a reduced growth rate. The doubling time of RES167 increased from 3.6 to 4.2 h when compared with a non-induced culture. Obviously, the six rrn operons were stringently regulated in the rel mutant RES167 (Fig. 2B) when compared with the non-induced controls (Fig. 2A). The extent of the respective responses is a similar effect of a 1.3-fold decrease of the amount of Cat protein. In Streptomyces coelicolor the accumulation of (ppp)Gpp after serine hydroxamate addition was also accompanied by a marked reduction of rrnD transcription and a simultaneous decrease in growth rate [13]. In E. coli, all seven rrn operons were stringently regulated exhibiting a more extensive 1.9–3-fold decrease in cat expression upon serine hydroxamate-induced amino acid starvation [13]. This effect is based on decreased transcription initiation at susceptible rrn promoters and is mediated through the accumulation of (ppp)Gpp, which is synthesized by the RelA protein.

Since (ppp)Gpp synthesis is abolished in C. glutamicum RES167Δrel [6], no (ppp)Gpp accumulation can occur upon amino acid starvation. In addition, no response to serine hydroxamate-induced amino acid starvation was measured in the rel mutant RES167Δrel (Fig. 2B). The amount of Cat protein was indistinguishable from that determined in non-induced cultures (Fig. 2A) and thus, the C. glutamicum strain RES167Δrel displayed a relaxed control of rrn expression. Therefore, the use of rrn–cat fusions provided experimental evidence that (ppp)Gpp is the effector molecule of the stringent response regulating the rrn operon expression in C. glutamicum.

Fig. 3. Effect of a deleted rel gene on the growth of C. glutamicum in minimal medium. (A) Effect of rel inactivation in C. glutamicum RES167Δrel (∇). The rel mutant RES167 (●) was used as control. (B) Complementation of C. glutamicum RES167Δrel (●) with a plasmid-encoded copy of rel on pLW67. C. glutamicum RES167 carrying the cloning vector pEBM2 (○) was used as control. (C) Effect of medium supplementation with the amino acid mixture casamino acids on the growth of C. glutamicum RES167 (●) and C. glutamicum RES167Δrel (∇). In addition, the growth of C. glutamicum RES167 (●) and C. glutamicum RES167Δrel (○) was assayed in mineral medium supplemented with histidine and serine. The data are means of three independent growth assays.

3.2. Amino acid requirement of a rel mutant strain of C. glutamicum

E. coli strains lacking (ppp)Gpp showed several phenotypic changes including multiple amino acid auxotrophies [2]. To assess the effect of a deleted rel gene on the growth of C. glutamicum, the strains RES167 and RES167Δrel were grown in liquid mineral medium (Fig. 3A). Obvi-
ously, the defined rel mutant C. glutamicum RES167drel grew more slowly in mineral medium when compared to the relI control C. glutamicum RES167. Growth of RES167drel was completely restored after introducing a plasmid-encoded rel gene on vector pLW67 (Fig. 3B) which consists of the cloning vector pEBM2 [16] and the 4.1-kb Scal–HindIII rel-carrying fragment of pLW65 [6]. Since the only genetic difference between RES167 and RES167drel is the defined rel deletion, these experiments strongly suggest that the rel genotype in the chromosome of C. glutamicum is responsible for growth deficiency.

Further analyses in mineral medium showed that the growth of RES167drel can be restored by supplementing the minimal medium with the amino acid mixture casamino acids (Fig. 3C). To analyze this requirement in more detail, a set of 20 minimal media, containing a full complement of amino acids with a single omission (‘20 aa minus X test’ [2]), and control media containing none or a complete complement of amino acids were inoculated with RES167 and RES167drel. The growth of both strains was monitored after 16 h of incubation at 30°C by measuring the OD580 nm of the cultures. Amino acid requirements for a strain are indicated by poorer growth than in the control medium containing the complete set of amino acids [2]. Although the parental strain RES167 is prototrophic, the ‘20 aa minus X test’ displayed a requirement of RES167drel for the amino acids histidine and serine. Since the growth of the control strain RES167 was not affected upon omission of histidine and serine, it can be excluded that the amino acid supplementation created metabolic imbalances preventing growth. The result of the ‘20 aa minus X test’ was confirmed in a further growth assay which showed that RES167drel is able to grow in liquid mineral medium supplemented with the amino acids histidine and serine (Fig. 3C). These data clearly demonstrate that the defined rel deletion in the chromosome of C. glutamicum RES167 resulted in an amino acid requirement for histidine and serine.

In contrast to the observation made in the C. glutamicum rel mutant, the auxotrophy for amino acids in E. coli relA–spoT double mutants is more complex and revealed host-specific differences [2]. Some requirements exist in common in all E. coli host backgrounds investigated. Besides histidine and serine these include arginine, methionine, and phenylalanine, and probably the valine isoleucine, leucine, and threonine group of amino acids. In addition, some E. coli strains revealed a strong requirement for glycine and lysine whereas others require tyrosine but not glycine or lysine [2]. Likewise, the relA mutant of B. subtilis displayed a strong requirement for the amino acid valine and a weaker one for isoleucine, leucine and methionine [3]. Insertion mutants of S. equisimilis grow slowly on both a chemically defined medium supplemented with amino acids and on complex media [17]. A multiple amino acid requirement was not reported for the relA mutant of S. coelicolor, but a residual level of (pppGpp synthetase activity was observed in the disruption mutant [18].

Of the amino acid requirements identified in C. glutamicum, the one for histidine is most interesting with respect to improved amino acid production. It has been suggested that the histidine biosynthesis is under positive control during the stringent response [1]. Maximal expression levels of the histidine operon from Salmonella typhimurium were strongly dependent on the addition of pppGpp to a coupled in vitro transcription–translation system [19]. Additional studies in S. typhimurium implied that (pppGpp acts as a positive effector molecule of histidine operon expression through an attenuator-independent mechanism which was localized at the level of promoter activity [20]. Therefore, genetic examination of a putative effect on his gene expression mediated by (pppGpp at the his promoter region in C. glutamicum may eventually result in strains with improved histidine production. Further experiments on the expression level of histidine biosynthesis genes will help to understand this regulatory mechanism in C. glutamicum.

References


