The role of the *Corynebacterium glutamicum* *rel* gene in (p)ppGpp metabolism

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To investigate the metabolism of (p)ppGpp in amino-acid-producing coryneform bacteria, a PCR-based strategy using degenerate consensus oligonucleotides was applied to isolate the *rel* gene of *Corynebacterium glutamicum* ATCC 13032. The gene consists of 2283 nucleotides and encodes a protein of 760 amino acids with a molecular mass of 84.4 kDa. The amino acid sequence revealed extensive similarities to the related proteins RelA and SpoT of *Escherichia coli*, which are known to be involved in (p)ppGpp biosynthesis and degradation. The *C. glutamicum rel* gene is located downstream of the *apt* gene encoding an adenine phosphoribosyltransferase, and an ORF with similarities to *dciAE*, which represents part of a dipeptide transport system in *E. coli*. A *C. glutamicum* mutant strain carrying a defined deletion in the *rel* gene was constructed. This mutant failed to accumulate (p)ppGpp in response to amino acid starvation. When overexpressed in *E. coli*, the *C. glutamicum rel* gene was able to reverse growth defects caused by an overexpressed *relA* gene. It is proposed that the *C. glutamicum rel* gene encodes a bifunctional enzyme with (p)ppGpp synthetase and (p)ppGpp-degrading activities.

Keywords: stringent response, (p)ppGpp, *Corynebacterium*, *relA*, *spoT*

INTRODUCTION

In *Escherichia coli*, amino acid starvation leads to pleiotropic metabolic alterations, termed the stringent response, mediated by the highly phosphorylated guanine nucleotides ppGpp and pppGpp [collectively abbreviated (p)ppGpp]. Accumulation of (p)ppGpp is also observed after other nutrient or environmental stresses, such as carbon source, nitrogen or phosphate limitation. This indicates that (p)ppGpp also plays a central role in growth rate control. An elevated level of (p)ppGpp results in complex changes in the pattern of gene expression. While transcription of many genes and most stable RNA species is rapidly reduced, expression of stationary-phase-specific genes is induced and the expression of certain anabolic genes, for example amino

acid biosynthetic genes, is increased (reviewed by Cashel *et al.*, 1996; Codon *et al.*, 1995). In addition, it has been shown that the stringent response is correlated with increased glutamate excretion in *E. coli* (Burkovski *et al.*, 1995).

The related proteins RelA and SpoT are involved in the synthesis of (p)ppGpp in *E. coli*. The RelA protein, responsible for (p)ppGpp biosynthesis during amino acid deprivation, is a ribosome-associated enzyme catalysing the transfer of a pyrophosphoryl group of ATP to the 3'-hydroxyl position of GTP or GDP. The enzyme is active when uncharged codon-specific tRNAs bind to the ribosomal A-site (Cochran & Byrne, 1974; Goldman & Jakubowski, 1990). The SpoT protein was originally identified as a (p)ppGpp 3'-pyrophosphohydrolase responsible for degrading (p)ppGpp to GTP or GDP and pyrophosphate (An *et al.*, 1979). However, the presence of (p)ppGpp in *relA* mutant strains (Hernandez & Bremer, 1991), the absence of all detectable (p)ppGpp in *relA/spoT* double mutants (Xiao *et al.*, 1991) and the fact that RelA and SpoT have extensive amino acid sequence similarity throughout their length (Metzger *et al.*, 1989) led to the proposal that SpoT is a bifunctional

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Abbreviation: PEI, polyethyleneimine.

The GenBank accession number for the 4.08 kb *rel* region reported in this paper is AF038651.

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic	Source or reference
<i>C. glutamicum</i>		
ATCC 13032	Wild-type strain, Nx ^R	ATCC
RES167	Restriction-deficient mutant of ATCC 13032, Δ <i>cglRI cglRII</i>	Universität Bielefeld
RM4	Restriction-deficient mutant of ATCC 13032 obtained by chemical mutagenesis	Universität Bielefeld
ASC30	RES167 carrying pASC20 integrated in the chromosome	This work
RES167 Δ <i>rel</i>	<i>rel</i> deletion mutant of RES167	This work
RM4 Δ <i>rel</i>	<i>rel</i> deletion mutant of RM4	This work
<i>E. coli</i>		
DH5 α MCR	F ⁻ <i>supE44 endA thi-1 recA1 gyrA96 relA1 deoR</i> Δ (<i>lac-argF</i>)U196 ϕ 80d <i>lacZ</i> M15 <i>mcrA</i> Δ (<i>mmr hsdRMS mcrBC</i>)	Grant <i>et al.</i> (1990)
S17-1	<i>hsdR pro recA</i> carrying RP4-2-Tc::Mu in the chromosome	Simon <i>et al.</i> (1983)
CF1648	Wild-type MG1655	Xiao <i>et al.</i> (1991)
CF1652	CF1648 Δ <i>relA251</i>	Xiao <i>et al.</i> (1991)
Plasmids		
pK18 <i>mob</i>	Mobilizable cloning and sequencing vector, Km ^R	Schäfer <i>et al.</i> (1994)
pTrc99A	pKK233-2-derivative, <i>trcP</i> , <i>lacIⁿ</i> , pUC18 polylinker, Ap ^R	Amann <i>et al.</i> (1988)
pASC20	pK18 <i>mob</i> carrying an 140 bp PCR amplificate of the <i>C. glutamicum rel</i> gene	This work
pASC21	pK18 <i>mob</i> with a 2.2 kb <i>EcoRI</i> insertion containing the 5' region of <i>rel</i>	This work
pASC22	pK18 <i>mob</i> with a 2.7 kb <i>HindIII</i> insertion containing the 3' region of <i>rel</i>	This work
pASC25	pK18 <i>mob</i> carrying a 1.8 kb <i>XhoI</i> PCR amplificate of <i>rel</i>	This work
pASC26	pASC25 lacking a 400 bp <i>SalI</i> fragment	This work
pLW60	pK18 <i>mob</i> containing an 800 bp <i>EcoRI</i> - <i>BamHI</i> insert originating from pASC21	This work
pLW65	pK18 <i>mob</i> carrying a 4.75 kb <i>HindIII apt-rel</i> region of <i>C. glutamicum</i>	This work
pLW68	pK18 <i>mob</i> harbouring a 3.5 kb <i>XhoI</i> - <i>HindIII rel</i> region originating from pLW65	This work
pSFS10	pTrc99A harbouring <i>relA</i> downstream of the <i>trc</i> promoter	This work

enzyme, responsible for the second source of activity for the formation of (p)ppGpp (Murray & Bremer, 1996). A mutational analysis of the *spoT* gene revealed that the two catalytic sites of SpoT are located in distinct but overlapping regions of the N-terminal region (Gentry & Cashel, 1996). The synthetase activity of SpoT is active during energy exhaustion and provides a basal level of (p)ppGpp during exponential growth (Ryals *et al.*, 1982).

In contrast to the situation described for *E. coli*, a chromosomal deletion in the *Bacillus subtilis relA* gene eliminates (p)ppGpp synthesis in response to both amino acid deprivation and glucose exhaustion (Wendrich &

Marahiel, 1997). Moreover, a mutant strain of *Streptococcus equisimilis* H46A, carrying an insertion in a *relA/spoT* homologous gene, termed *rel*, fails to accumulate (p)ppGpp in response to amino acid starvation and was shown to encode an additional (p)ppGpp hydrolase activity. These findings support the idea that in some bacteria one gene mediates the functions that reside in separate *E. coli* genes (Mechold *et al.*, 1996).

The Gram-positive, nonsporulating soil micro-organism *Corynebacterium glutamicum* is of special interest for the industrial production of amino acids, nucleotides and numerous metabolites (Wohlleben *et al.*, 1993). In the last decade, genetic tools have been developed for

Fig. 1. Restriction map of the contiguous 4.75 kb *apt-rel* region from *C. glutamicum* ATCC 13032. The positions of the ORFs (*dciAE*, *apt*, *rel*, *orf 4*) are indicated by arrows and the position of the transcriptional terminator of *orf 4* is marked (Ω). The location of the 140 bp PCR product (ASC20) and the 800 bp *EcoRI*-*Bam*HI fragment upstream of *apt* used for the isolation of the entire *rel* gene (LW60) are shown under the restriction map. Also indicated are the fragments used for the construction of a chromosomal *rel* deletion (ASC25, ASC26). For this purpose, a 1.8 kb internal fragment of the *apt-rel* region was amplified from the *C. glutamicum* chromosome (ASC25) and cloned into pK18*mobsacB*. The resultant plasmid, pASC25, was cut with *SalI* and religated, to form plasmid pASC26; this carries a modified *apt-rel* fragment which lacks a central 400 bp *SalI* fragment (ASC26).

this group of bacteria and a variety of genes involved in amino acid biosynthesis have been cloned. Since then, the synthesis of selected amino acids has been improved by genetic engineering and amplification of relevant biosynthetic genes, resulting in highly productive strains – so-called amino acid hyperproducers (Sahm *et al.*, 1995). A recent study in *C. glutamicum* has pointed out that, as in *E. coli*, RNA synthesis as well as growth rate are inversely correlated with the cellular ppGpp concentration (Ruklisha *et al.*, 1995). Therefore, knowledge about the genetic background of (p)ppGpp metabolism in *C. glutamicum* could possibly lead to a better understanding of the stringent response in these bacteria.

In the present study we report the identification and molecular characterization of a *C. glutamicum* gene, termed *rel*, which shows significant homology to *relA* and *spoT* genes. (p)ppGpp accumulation assays in wild-type and in *rel* mutant strains, as well as expression studies of the *rel* gene in *E. coli*, provide considerable evidence that *rel* is bifunctional, encoding a (p)ppGpp synthetase as well as a (p)ppGppase.

METHODS

Bacterial strains, plasmids and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *C. glutamicum* strains were routinely grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 37 °C and 30 °C, respectively. Antibiotics used for plasmid selection were kanamycin (50 µg ml⁻¹ for *E. coli*, 25 µg ml⁻¹ for *C. glutamicum*) and ampicillin (100 µg ml⁻¹ for *E. coli*).

DNA manipulation and DNA transfer. Plasmid DNA from *E. coli* was isolated by the alkaline lysis technique (Sambrook *et al.*, 1989). Total chromosomal DNA of *C. glutamicum* was prepared as described by Tauch *et al.* (1995). DNA restriction, agarose gel electrophoresis, Klenow treatment and ligation were carried out as described by Sambrook *et al.* (1989). Enzymes for DNA manipulation were purchased from Pharmacia or Boehringer. DNA restriction fragments required for cloning were isolated from agarose gels using the US BioCleanMP Kit (United States Biochemicals). All cloning

procedures were carried out using *E. coli* DH5 α MCR (Grant *et al.*, 1990). Plasmid DNA was introduced into *E. coli* strains by electroporation (Tauch *et al.*, 1994). Intergeneric conjugal transfer of plasmids from *E. coli* to *C. glutamicum* followed the mating procedure described by Schäfer *et al.* (1990). Transconjugants were selected on LB agar containing 50 µg nalidixic acid ml⁻¹ and 25 µg kanamycin ml⁻¹.

Polymerase chain reactions. The synthetic oligonucleotides 5'-GACCG(GT)TT(GA)CATAA(CT)ATGCG and 5'-GAA-AG(CA)CAG(AG)TCTTC(TC)AA (nucleotides in parentheses indicate positions of degeneracy) were used for the amplification of a 140 bp internal fragment of the *C. glutamicum rel* gene with *Taq* DNA polymerase from Gibco-BRL. The promoterless *E. coli relA* gene was amplified with the PCR primers 5'-ACTGGCGATGCTGGATATGT and 5'-GATTGAGCGCCTGCATTAAC using *Pfu* DNA polymerase (Stratagene). PCR experiments were carried out with a PCT-100 thermocycler (MJ Research). Initial denaturation was conducted at 94 °C for 2 min followed by 90 s denaturation, 90 s annealing at $T_{4(\text{primer 1 or 2})} - 5$ °C (Suggs *et al.*, 1981) and extension at 72 °C for 90 s (*Taq* polymerase) or 6 min (*Pfu* polymerase), respectively. This cycle was repeated 30 times followed by a final extension step for 10 min at 72 °C. PCR products were purified using the Qiagen PCR Purification Spin Kit from Qiagen.

Southern hybridization. Chromosomal DNA of *C. glutamicum* was digested with restriction enzymes and separated on 0.8% agarose gels. Southern blots were prepared on Hybond-N nylon membranes (Amersham) using the vacuum blotter Vacu-Gene (Pharmacia). Fixation of DNA, labelling of DNA probes and hybridization were performed with the non-radioactive DIG DNA Labelling and Detection Kit from Boehringer.

DNA sequencing and sequence analysis. Unidirectional deletions were introduced into the plasmid inserts of pASC20 and pASC21 using the Double Stranded Nested Deletion Kit (Pharmacia). DNA sequencing analysis was performed with DNA isolated and purified using the Qiagen Plasmid Mini Kit (Qiagen) by the dideoxy chain-termination method of Sanger *et al.* (1977). The sequencing reactions were carried out with the AutoRead Sequencing Kit from Pharmacia. Electrophoretic analysis and detection of the sequenced products

Rel_C.glu	-----MSLERNT	7
SpoT_M.lep	-----MADDQGTAAQLQPVQVPGPAVEAPETPVETL	32
RelA_S.coe	MPDEAQPLTAAPKESASASAAKPAAPSAPQAKNDTHGPIQHAPAAPVDKPAEQPRPKPLPAERPQNAFVVRAPAG	75
Rel_S.eqi	-----	
Rel_C.glu	QKS-SMGVRSMSARLARSITGNRVRTNPFVLDPLLSIHRQFHP---RADVQVLERAYDTAERLHDGVIRKSGDFYI	78
SpoT_M.lep	KTS-SSASRRVRARLARMTAQRSTISPVLEPLVAVHKEFP---KANLSIVQRAFEVADQRHASQLRRSGDFYI	103
RelA_S.coe	QPARGSSNRVRARLARLGVQRANPNPNVLEPLLRIVRGNDPKIETSTLRQIERAYQVAERWHRGQKRKSGDFYI	150
Rel_S.eqi	-----MAKEINLTGEEVVALAAKYMN---ETDAAFVKKALDYATAAHFYQVRKSGDFYI	51
		
Rel_C.glu	THPLAVATIAAEIGMDTTTLVAALLDVTEDTDSLDLTDTRDFGEEVARLVGGVTKLDKVALG---AAAEAEITRK	151
SpoT_M.lep	THPLAVANILAEIGMDITTLVAALLDVTEDTGYTLEALSEEFQDEVGHLVDGVTKLDRVVLG---SAAEGETIRK	176
RelA_S.coe	THPLAVTTILAEIGMDPATLMAGLLDVTEDTGYLEDLRRDFGDDVTLVDGVTKLDRVVKFG---EAAQAEITVRK	223
Rel_S.eqi	VHPIQVAGILADLHLDAVTACGFLEDVVEDTDTITLDNIEFDGKDVDRDVGVTGLKGVKEYKSHEEQLAENHRK	126
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Rel_C.glu	MIIVAMSQDPRLVLVIKADRLHNMRTMRFPPKQAKKARQTLVIAPLAHLRGLMASVKWELEDLSFAILYPKKYE	226
SpoT_M.lep	MITAMARDPRVLVIKADRLHNMRTMRFPPKQAKKARETLEVIAPLAHLRGLMASVKWELEDLSFAILYPKKYE	251
RelA_S.coe	MVVAMAKDPRVLVIKADRLHNMRTMRYLKREKQEKKARETLEIYAPLAHLRGLMNTIKWELEDLAFAILYPKMY	298
Rel_S.eqi	MLVAMSKDIRVILVKADRLHNMRTLKLHKKQERISRETMEIYAPLAHLRGLSRIKWELEDLAFRILNETEFY	201
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Rel_C.glu	EIVRLVADRAPSRRDYLKEIIDQVTGGLRENNIAAGVLGRPKHYWSIYQKMIIVGRDRDFDIFDLVGIRILVDNVN	301
SpoT_M.lep	EIVRLVAGRAPSRRDYLAKVRAEIIITLGLASKIKATVEGRPKHYWSIYQKMIIVGRDRDFDIDHDLVGIRILCDEIR	326
RelA_S.coe	EIVRLVAERAPKRDEYLAVVTDEVQDLRAARIKATVTGRPKHYWSIYQKMIIVGRDRDFDIDHDLVGIRILVDNVR	373
Rel_S.eqi	KISHMMNEKRREALVDDIVTKIKSYTTEQGLFGDVYGRPKHYWSIYQKMIIVGRDRDFDIDHDLVGIRILVDNVR	276
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Rel_C.glu	NCYAAIGVVHSLFNALPGRFKDYISAPRFGVYQSLHTTVMGPGGKPLEVQARTHDMHYNAEFGIAAHWRYKETKG	376
SpoT_M.lep	DCYAAIGVVHSLWQPMAGRFKDYIAQPRYGVYQSLHTTVMGPGGKPLEVQIRTRDMHTAEYGIAGAHWRYKEAKG	401
RelA_S.coe	DCYAAIGVTHARWNPVGRFKDYIAMPKFNMYQSLHTTVMGPGGKPLEVQIRTFDMHRAEYGIAGAHWRYKEAV	448
Rel_S.eqi	DVYAMVGYIHELWRPMPGRFKDYIAAPKANGYQSIHTTVYGPKG-PLEIQTIRKEMHQAHYGVAAHWAHKKGV	350
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Rel_C.glu	-----SHSGEQAQEVDMAMMRQLLDWQKEAA-DPNEFLDSLRYDLTSKQIFVFTPKQDVVNLFVNST	437
SpoT_M.lep	R-----NGVLHPHAAAEIDDMAMMRQLLDWQKEAA-EPGEFLESRLRYDLAVQEIFVFTPKQDVVLTFTGST	466
RelA_S.coe	AGASKVRTDAPKSSGKSKDDHLNDMAWLRLQLLDWQKETE-DPGEFLESRLRFDLSRNEVFTVFTPKQDVVIALFAGAT	522
Rel_S.eqi	-----GKVNQAEQKVMNWKELVELQDASNGDAVDVDSVKEDIFSERIYVFTPTGAVQELPKDSG	412
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Rel_C.glu	PVDFAYAVTEVGHRCIGAKINGKLVALETKLSQDRVEVFTSKDQNAQPSRGWQEFVVSAPRAKAKIRQWFAKER	512
SpoT_M.lep	PVDFAYAVTEVGHRCIGARVNGRLVALERKLENGEFVFTSKAPNAGPSRDWQFVVSAPRAKAKIRQWFAKER	541
RelA_S.coe	PVDFAYAVTEVGHRTIGARVNGRLVPLESTLDNGDLVEVFTSKAAGAPSRDWLGFVKSAPRAKAKIRQWFSKER	597
Rel_S.eqi	PIDFAYAIETQVGEKAIGAKVNGRMVPLTAKLKTGDVVEIVTNPN-SFGPSRDWIKLVKTNKARNKIRQFFKNQD	486
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Rel_C.glu	REEYLEAGRDALAAVIQRGGLPMHRLFTASSMKTVA TELHYPDVDALYTAIGSGSVSAQHVVNRLMAIFGDEED-	586
SpoT_M.lep	REEALEAGRDAMAREVRRGGLPLQRLVNGESMAAVARELHYIDVSALYTAIGEGHVSARHVVQRLLAELGGIDQ-	615
RelA_S.coe	RDEAIEQKQDAIVRAMRKQNLPIQRIITGDSLVTLAHMRYSDISALYAAIGEGHVSAPNIVQKLVLQALGGEEA-	671
Rel_S.eqi	KELSVNKGKMDLVSYFQEQGYVANKYLDKKRIEAILPKVSVKSEESLYAAVGFQDISPVSVFNKLTEKERREER	561
		
Rel_C.glu	-----AEDALVARTPFSELVNSRATTESSTGILVEGSPDVMAKLAKCCPFVGGDEIFGFTVTRGGVSVERTICIN	656
SpoT_M.lep	-----AEEELAERSTPTMLR-RQRSTDVGVSVGAPGVLTAKLAKCCPFVGGDAIMGFTVTRGGVSVERTICIN	684
RelA_S.coe	-----ATEEIDESVPPSRGRGRKRANADPGVVVGVEDVWVKLAKCCPFVGGDPIIGFTVTRGGVSVERTICIN	741
Rel_S.eqi	AKAKAEAEELVNGGEIKHENKDVLRSENGVVIQCGASGLLMRIAKCCPFVGGDPIEGYITKGGLAIALRALCIN	636
		
Rel_C.glu	VEKLKEEPERIVSVSWASEGQGSVFSATLQLEALDRAGLLFELTRVINEQKVSVTAMNSHCSGD-RVATVRFTFA	730
SpoT_M.lep	TASLQQQAERIIEVLWAPSSS-SVFLVAIQVEALDRHRLLLDITRALADERVDILSASVTTSGD-RVAISRFTFE	757
RelA_S.coe	VDSLREPERILEVEWAPTQS-SVFLVAIQVEALDRSRLSDVTRVLSQHVNLISAAVQTSRD-RVATSRFTFE	814
Rel_S.eqi	IKSQDGYQERILEVEWDLNSSSKDYAEIDYGLNRRGLLDVQLISNSTKSISTVNAQPTKMKFANHVSFG	711
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Rel_C.glu	VSDTKQLGSLMTQLRNAEGVFDVYRVTSGG---	760
SpoT_M.lep	MGDPKHLGHLNLRVNRVGVYDVYRVMSAS---	787
RelA_S.coe	MGDPKHLGHVLRKAVRGVGVYDVYRVTSARRPS	847
Rel_S.eqi	IPNLTHLTTVVEKIKAVPDVYSVKRTNG----	739
		

Fig. 2. Multiple alignment of the deduced amino acid sequence of the *C. glutamicum* Rel protein with homologues from other bacteria. Identical amino acids are typed in bold and marked by asterisks; a dot represents positions with conservative substitutions. The aligned cysteine, glycine and histidine residues in a conserved C-terminal region, possibly acting as a metal-binding domain, are boxed. C.glu, *C. glutamicum*; M.lep, *M. leprae* (EMBL U00011); S.coe, *Streptomyces coelicolor* A3(2) (Chakraborty et al., 1996); S.eqi, *S. equisimilis* H46A (Mechold et al., 1993).

were accomplished with the help of an ALF DNA sequencer from Pharmacia. Computer-assisted compilation of the DNA sequences was done with the computer programs of Staden (1986) and Pearson & Lipman (1988). Searches for amino acid similarities were carried out with the BLAST e-mail service (Altschul *et al.*, 1990). Multiple alignments were computed by the CLUSTAL W program (Thompson *et al.*, 1994).

Construction of *rel* deletion mutants of *C. glutamicum* RES167 and RM4. Defined chromosomal deletions in the *rel* gene of *C. glutamicum* RES167 and RM4 were constructed using the *sacB* system, which helps to identify an allelic exchange by homologous recombination (Schäfer *et al.*, 1994). For this purpose, a 1.8 kb internal fragment of the *apt-rel* region was amplified from the *C. glutamicum* chromosome using PCR with the oligonucleotides ASC25-1 (5'-CTTGAATACGGC-CTGCA) and ASC25-2 (5'-TCTTGGCCTTTGCTCGAG). The PCR primers ASC25-1 and ASC25-2 were deduced from the known nucleotide sequence (ASC25-1, nt 582–599, ASC25-2, nt 2358–2341). The downstream primer ASC25-2 contained an A2345 to T exchange leading to an *XhoI* site. The purified 1.8 kb amplicon was cleaved with *XhoI* and cloned into the *SalI*-digested *E. coli* vector pK18mob*sacB* (Schäfer *et al.*, 1994), yielding pASC25 (Fig. 1). Subsequently, pASC25 was cut with *SalI* to delete an internal 400 bp fragment (corresponding to amino acid residues 129–266) of the *C. glutamicum rel* gene and religated. The resulting plasmid pASC26 (Fig. 1) was transferred to *E. coli* S17-1 and mobilized to *C. glutamicum* RES167 and RM4 by conjugation. The plasmid can only establish itself by homologous recombination into the chromosome in such a way that the resulting *C. glutamicum* strains carried the modified *apt-rel* region and the wild-type genes separated by vector sequences. Excision of the plasmid can be selected for by growing the cells on LB agar containing 10% sucrose. Cells able to grow on this medium have lost the plasmid due to a second cross-over event that either restores the wild-type gene arrangement or leads to a mutant strain carrying a defined deletion and a frame shift mutation in the chromosome. Southern hybridizations using *HindIII*-digested chromosomal DNAs of sucrose-resistant clones and labelled pASC25 DNA were performed to distinguish between both genomic situations and to verify the construction of the chromosomal *rel* deletion.

Measurement of (p)ppGpp accumulation. Strains were screened for patterns of (p)ppGpp accumulation using the rapid screening method described by Cashel (1994). Non-uniformly $^{32}\text{P}_i$ -labelled *E. coli* and *C. glutamicum* cells were obtained by growing the strains overnight on LB plates, followed by dispersion of single colonies in phosphate-free MOPS minimal medium and incubation with MOPS medium containing $10\ \mu\text{Ci}\ ^{32}\text{P}_i\ \text{ml}^{-1}$ ($3.7 \times 10^5\ \text{Bq}\ \text{ml}^{-1}$) for 30 min at 37 °C. For amino acid starvation responses, the colonies were resuspended in amino-acid-free, phosphate-free MOPS medium supplemented with DL-serine hydroxamate (1 mg ml $^{-1}$) and L-valine (0.5 mg ml $^{-1}$). The samples were then mixed with 13 M formic acid and three freeze-thaw cycles were performed. Afterwards, the samples were centrifuged and 5 μl samples of supernatant were subjected to one-dimensional polyethyleneimine (PEI) TLC developed with 1.5 M potassium phosphate (pH 3.4). Nucleotide abundance was quantified by PhosphorImager analysis (Fuji BAS1000). Amounts of ppGpp and pppGpp were expressed as fractions of the total activity of blank-corrected GTP, ppGpp and pppGpp. The nucleotide identities were verified by cochromatography with standards after two-dimensional PEI-TLC in 3.3 M ammonium formate/4.2% ammonium borate (pH 7) (first dimension) and 1.5 M KPi (pH 3.4) (second dimension).

RESULTS

Cloning of a *C. glutamicum* DNA fragment carrying sequences homologous to internal fragments of *relA* and *spoT* genes of other bacterial species

We used a PCR-based strategy to clone *relA/spoT* homologous genes from *C. glutamicum*. A sequence alignment of *spoT* from *Mycobacterium leprae* (EMBL 00011) and the *rel* gene from *Streptococcus equisimilis* (Mechold *et al.*, 1993) revealed several conserved nucleotide regions. These regions were chosen for the design of degenerate oligonucleotides acting as primers in PCR experiments with chromosomal DNA from *C. glutamicum* as a template. One primer pair led to an amplified product of approximately 140 bp corresponding to the size expected for a *relA*- or *spoT*-like gene fragment (ASC20 in Fig. 1). The PCR product was treated with Klenow polymerase and cloned in *SmaI*-cleaved pK18mob, yielding pASC20. The determination of the DNA sequence of the pASC20 140 bp insert using standard sequencing primers revealed homology to internal fragments of *relA*- and *spoT*-related genes. Its origin from *C. glutamicum* was verified by using pASC20 as probe in a Southern blot experiment with *C. glutamicum* chromosomal DNA (data not shown).

The missing 5' and 3' regions of the gene were isolated by applying the plasmid rescue technique. Plasmid pASC20 was introduced into *E. coli* S17-1 by electroporation and mobilized into *C. glutamicum* RES167. The plasmid can only establish itself by integration into the chromosome via homologous recombination, yielding the strain *C. glutamicum* ASC30. Specific integration was confirmed by Southern hybridization with pASC20 as a probe (data not shown). To isolate adjacent chromosomal fragments, total DNA of *C. glutamicum* ASC30 was isolated, cut with *EcoRI* (5' region) and *HindIII* (3' region) and finally religated. The ligation mixtures were subsequently transformed into *E. coli* DH5 α MCR. The resulting plasmids pASC21 and pASC22 carried insertions of approximately 2.2 kb and 2.7 kb, respectively. Both plasmids are overlapping by the 140 bp fragment used for integration and therefore represent a contiguous 4.75 kb DNA region of the *C. glutamicum* chromosome, of which a restriction map is shown in Fig. 1.

DNA sequence analysis of the isolated DNA region identified the putative *C. glutamicum rel* gene

The sequence of a 4077 bp *ScaI-HindIII* region was determined, represented by a 1.5 kb *ScaI-HindIII* fragment of the pASC21 insert and the complete 2.7 kb *EcoRI-HindIII* insertion of pASC22. The overall G+C content of the sequenced fragment is about 53 mol%, which agrees well with the range of 51.2–54.4 mol% reported for the *C. glutamicum* chromosome (Malumbres *et al.*, 1993). A computer-assisted analysis of the coding probability (Staden, 1986) revealed four ORFs with protein coding character (Fig. 1).

Fig. 3. Accumulation of (p)ppGpp in *C. glutamicum* wild-type and *rel* deletion mutants in response to amino acid starvation. Lanes: 1, *E. coli* CF1648, wild-type; 3, *C. glutamicum* ATCC 13032, wild-type; 4 and 6, RES167 and RM4, restriction-deficient derivatives of ATCC 13032; 2, CF1652, $\Delta relA$; 5 and 7, Δrel hosts of RES167 and RM4. Cultures were non-uniformly labelled with ^{32}P , and amino acid starved after growth on LB plates, as described in Methods. The positions of standards after PEI-TLC development in 1.5 M KH_2PO_4 are indicated on the right. *C. glu*, *C. glutamicum*.

The largest ORF within this region, ORF 1 (nt 849–3131), encodes a protein of 760 amino acid residues with a calculated molecular mass of 84.374 kDa and a deduced isoelectric point of 6.53. Eight nucleotides upstream of the putative ATG start codon a sequence (AAAGGCGG) that is complementary to the 3' end of the 16S rRNA of *C. glutamicum* was detected (J. Kalinowski, unpublished data), probably acting as a ribosome-binding site. A putative promoter region resembling the consensus –35 and –10 sequences of *E. coli* and *C. glutamicum* promoters (Hawley & McClure, 1983; Patek *et al.*, 1995) is located 69 nucleotides upstream of the ATG start codon. Sequence comparison of the deduced ORF 1 protein revealed significant homology to RelA and SpoT equivalents from other bacteria (Fig. 2), including *Streptomyces coelicolor* (Chakraborty *et al.*, 1996) and *Streptococcus equisimilis* (Mechold *et al.*, 1993), displaying 73% and 65% homology, respectively. The highest degree of homology was found to SpoT of *M. leprae* (EMBL U00011) with 82% similar amino acid residues. Of particular significance was the alignability of three cysteines, a histidine and several glycine residues in a conserved C-terminal segment (boxed in Fig. 2), also appearing in *E. coli* SpoT (Sarubbi *et al.*, 1988) and in the RelA proteins of *E. coli* (Metzger *et al.*, 1988) and *Vibrio* sp. (Flårdh *et al.*, 1994). These residues are possibly involved in metal binding (Berg, 1986). The similarities of the nucleotide sequences as well as the amino acid sequences prompted us to term ORF 1 as the putative *C. glutamicum rel* gene.

ORF 2, which is located upstream of *rel*, consists of 558 nt (nt 264–821) and shows a G + C content of 53.8 mol%. A potential ribosome-binding site is located 9 nt upstream of the predicted translational startpoint. Computer-assisted searches did not provide typical promoter sequences upstream of its GTG start codon. ORF 2 encodes a protein of 185 amino acid residues with a calculated molecular mass of 19.531 kDa, a deduced isoelectric point of 4.49 and a high degree of similarity to adenine phosphoribosyltransferases (APT) from a variety of organisms. The most similar one found by database searches was the APT protein of *S. coelicolor*, showing 60% amino acid homology. In a salvage reaction less costly than *de novo* synthesis, APT catalyses the conversion of adenine and 5-phosphoribosyl pyrophosphate to AMP (Hershey & Taylor, 1986). Because of the high degree of sequence similarity, ORF 2 was termed *apt*.

ORF 3 is located upstream of *apt* and is not completely represented on the isolated fragment. It terminates at a TAG stop codon (nt 262–264) immediately in front of the *apt* start codon. Database searches displayed similarity to *dciAE* from *M. leprae* (41% identity and 64% similarity in 39 amino acids overlap); ORF 3 was accordingly named *dciAE*. The DciAE protein of *B. subtilis* and *E. coli* probably acts as a peptide-binding protein as part of the DciA protein complex, which forms a dipeptide transport system (Mathiopoulos *et al.*, 1991).

ORF 4, also not completely present on the sequenced fragment, is located downstream of *rel* and in the opposite orientation (nt 3691–4077). Comparison of the amino acid sequence deduced from ORF 4 with databases showed no significant similarity to known proteins. In the non-coding region between *rel* and ORF 4, a 32 nt inverted repeat is present, which may act as a *p*-independent transcriptional terminator.

A *C. glutamicum rel* mutant is unable to accumulate (p)ppGpp when starved for amino acids

To assess the role of the *C. glutamicum rel* gene in (p)ppGpp synthesis, we constructed *rel* mutants of the restriction-deficient wild-type derivatives *C. glutamicum* RES167 and RM4 using the pK18*mobsacB* vector system (Schäfer *et al.*, 1994) to select for rare homologous recombination events. (See Methods for a detailed experimental procedure of the strain construction.) The resulting strains *C. glutamicum* RES167 Δrel and RM4 Δrel , carry a modified gene that lacks a central 400 bp *SalI* fragment (corresponding to amino acid residues 129–266), resulting in a frame shift mutation ensuring its complete knock out.

We determined the effect of a non-functional *rel* gene on (p)ppGpp synthesis of the constructed *rel* mutant strains subjected to amino acid starvation. Cellular levels of (p)ppGpp were measured as described in Methods. Conditions of amino acid deprivation were imposed by the supplement of DL-serine hydroxamate, which in-

Fig. 4. Reversal of growth inhibition of *E. coli* due to the overexpressed *relA* gene by the overexpressed *rel* gene of *C. glutamicum*. Overnight cultures of *E. coli* CF1648, transformed with pSFS10 and either pK18mob (a) or pLW68 (b), were diluted 1:50 in fresh LBG medium and induced at OD₅₈₀ 0.15 by the addition of IPTG at a final concentration of 2 mM. Cultivations were done in 500 ml Erlenmeyer flasks with 100 ml LBG at 37 °C and 180 r.p.m., and aliquots for the determination of OD₅₈₀ were taken at the time intervals given. Values are means of at least three different experiments.

hibits tRNA^{Ser} aminoacylation, and L-valine, resulting in isoleucine starvation (Cashel *et al.*, 1996). The comigration of putative ³²P_i-labelled (p)ppGpp with unlabelled marker (p)ppGpp visualized by UV absorbance was verified by two-dimensional chromatography of *C. glutamicum* samples. As expected, under normal growth conditions none of the strains tested showed an accumulation of (p)ppGpp (data not shown). After induction of amino acid starvation we detected (p)ppGpp in nucleotide extracts of *C. glutamicum* ATCC 13032, RES167 and RM4, which comigrates with (p)ppGpp synthesized by the *E. coli* wild-type under similar conditions (Fig. 3). The spot corresponding to pppGpp is present only in relatively minor amounts in all three *C. glutamicum* strains, indicating that, as in *E. coli*, ppGpp is the major accumulation product. The apparently lower (p)ppGpp quantities in *C. glutamicum* relative to those of *E. coli* were probably due to the rapid screening method used in our experiments, since non-uniform nucleotide labelling is not linear either with time or with cell density (Cashel, 1994). In contrast, we observed no (p)ppGpp accumulation in *E. coli* CF1652 ($\Delta relA$) or in the *rel* mutant strains of RES167 and RM4 upon amino acid deprivation. These findings indicate that the synthesis of (p)ppGpp under amino acid starvation conditions in *C. glutamicum* depends on a functional *rel* gene.

***E. coli* growth defects caused by an overexpressed *relA* gene can be corrected in the presence of an overexpressed *C. glutamicum rel* gene**

After identification of *rel* as a gene encoding a functional (p)ppGpp synthetase, we addressed the question of whether there is a second orthologous gene functioning in (p)ppGpp breakdown. Attempts to identify a second *relA/spoT* homologue in *C. glutamicum* made use of heterologous hybridization experiments with 24 oligonucleotides designed according to highly conserved regions in SpoT of *E. coli* and *M. leprae* and the Rel proteins of *S. equisimilis* and *C. glutamicum*, using the preferred codons of corynebacteria (Malumbres *et al.*, 1993). Since these experiments (data not shown) gave no

evidence for the presence of a second *relA/spoT* homologous gene in *C. glutamicum*, the possibility arose that the *C. glutamicum rel* gene might substitute for both functions encoded by the *relA* and *spoT* genes in *E. coli*. This idea was supported by findings in *B. subtilis* (Wendrich & Marahiel, 1997) and *S. equisimilis* (Mechold *et al.*, 1996; Mechold & Malke, 1997), where most probably only one *relA/spoT*-like gene exists. Mutations affecting the SpoT (p)ppGpp hydrolase activity in *E. coli*, like the *spoT203* allele, which was obtained by hydroxylamine mutagenesis, result in a slow-growth phenotype due to elevations of (p)ppGpp levels (Xiao *et al.*, 1991). The massive overexpression of the *E. coli relA* gene in *E. coli relA⁺spoT⁺* strains results in 20- to 50-fold higher (p)ppGpp levels as compared to normal conditions of amino acid starvation and in a complete inhibition of growth (Schreiber *et al.*, 1991; Svitil *et al.*, 1993). The *spoT203*-dependent growth defect is abolished by coexpression of *spoT* due to its functional (p)ppGpp hydrolase activity, restoring a low (p)ppGpp level (Xiao *et al.*, 1991). Therefore, we analysed whether the overexpressed *C. glutamicum rel* gene is able to reverse the *E. coli* growth inhibition caused by strong overexpression of the *relA* gene, which would provide evidence for a (p)ppGpp-degrading function of the Rel protein.

The uninterrupted *rel* gene was isolated by using the plasmid rescue technique. For this purpose, an 800 bp *EcoRI*-*Bam*HI fragment located upstream of *apt* was inserted into pK18mob. After integration of the resulting plasmid pLW60 (Fig. 1) into the chromosome of *C. glutamicum* RES167, a *Hind*III rescue was carried out to obtain pLW65 with an 4.75 kb insert, representing the complete *apt-rel* region. Finally, a 3.5 kb *Xho*I-*Hind*III subfragment of the pLW65 insert was cloned into pK18mob, yielding pLW68, bearing *rel* under the control of the inducible *lac* promoter.

The complete *E. coli relA* gene, including the ribosome-binding site but without its own promoter, was amplified from the chromosome of *E. coli* CF1648 using PCR and cloned into *Sma*I-digested plasmid pTrc99A. The resulting vector pSFS10 carries a 2.5 kb fragment harbouring *relA* downstream of the IPTG-inducible *trc*

promoter. The functionality of the amplified *relA* gene was verified by its ability to abolish the SMG sensitivity of an *E. coli relA* strain (Uzan & Danchin, 1976). SMG sensitivity means that the growth of *E. coli relA* mutant strains, like DH5 α MCR (*relA1*), is prevented in minimal medium supplemented with the one-carbon-pool amino acids serine, methionine and glycine (SMG). When introduced into DH5 α MCR, pSFS10 enables growth of the strain on SMG plates (data not shown).

The *E. coli* strain CF1648 was transformed with pSFS10 (*E. coli relA*) and either pLW68 (*C. glutamicum rel*), or the control plasmid pK18mob. The effect of IPTG induction on growth of CF1648 containing the different pairs of plasmids was determined by measuring OD₅₈₀ of the growth medium. As shown in Fig. 4(a), induction of pSFS10 gives complete growth inhibition. In contrast, the presence of pLW68 abolishes growth inhibition (Fig. 4b), thereby suggesting a (p)ppGppase activity conferred by the *C. glutamicum rel* gene. Alternative explanations for the suppression of the *E. coli* growth defect, for example that the *E. coli* RelA synthetase activity could be inhibited by the *C. glutamicum* Rel protein, seemed to be less likely.

DISCUSSION

In *E. coli* two related genes, *relA* and *spoT*, function in the metabolism of the stringent response regulator (p)ppGpp. Since sequence information of *relA/spoT* homologous genes from several Gram-positive and Gram-negative bacteria was available, we applied a PCR-based strategy to isolate an equivalent from *C. glutamicum*. The entire *rel* gene was cloned by applying integration mutagenesis and the plasmid rescue technique. The deduced Rel protein shows strong similarities to all known RelA/SpoT orthologues. There is also a high degree of conservation in gene organization of the isolated region in bacteria related to *C. glutamicum*. In *C. glutamicum rel* is preceded by two ORFs that are similar to *apt*, encoding an adenine phosphoribosyltransferase (Hershey & Taylor, 1986), and *dciAE*, acting as a peptide-binding protein as part of a dipeptide transport system (Mathiopoulou *et al.*, 1991). The *apt* genes of *Mycoplasma genitalium* (Fraser *et al.*, 1995) and *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996) are located in front of their *spoT* genes, separated only by a small ORF of unknown function. In *Streptomyces coelicolor* A3(2) (Chakraborty *et al.*, 1996), *Bacillus subtilis* (Wendrich & Marahiel, 1997), *Mycobacterium leprae* (EMBL U00011) and *Mycobacterium tuberculosis* (Z77724), the *relA/spoT* homologues are preceded directly by *apt*-like genes. Additionally, the *dciAE* genes of *M. leprae* and *M. tuberculosis* are found immediately upstream of the respective *apt* genes. While the stringent response regions in Gram-positive bacteria appear extensively conserved, the corresponding regions of *relA* and/or *spoT* homologous genes in Gram-negative organisms, for example *E. coli* (Sarubbi *et al.*,

1988; Metzger *et al.*, 1988), *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Vibrio* sp. S14 (Flårdh *et al.*, 1994), are different from *C. glutamicum* and appear considerably more variable.

The analysis of the *C. glutamicum* Rel sequence failed to identify a putative ATP/GTP-binding domain. Moreover, neither in SpoT of *M. leprae* and Rel of *Streptococcus equisimilis*, nor in the Rel-like proteins of *E. coli* and *Vibrio* sp., is such a motif present. Only *Streptomyces coelicolor* A3(2) RelA contains a 'P-loop' consensus sequence probably involved in ATP/GTP-binding (amino acid residues 458–465) (Martinez-Costa *et al.*, 1996), which is surprising, since all enzymes involved in (p)ppGpp metabolism have to bind phosphate as a substrate for their catalytic activity. Nevertheless, a mutational analysis of SpoT from *E. coli* identified its amino residues 85–376 as being responsible for binding ATP, GTP or GDP (Gentry & Cashel, 1996). This corresponds to a region in the *C. glutamicum* Rel protein (residues 113–402) which shares a high degree of similarity to SpoT, providing evidence that this region of the Rel protein might be involved in binding of phosphate.

Since Rel of *C. glutamicum* is highly similar to both *E. coli* RelA and *E. coli* SpoT, it might represent a functional homologue of RelA, encoding a (p)ppGpp synthetase, or of SpoT, involved in (p)ppGpp degradation rather than synthesis. The *rel*-like genes of *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Mycobacterium leprae* and *Mycobacterium tuberculosis* were identified during genome sequencing projects and all termed *spoT*. However, this designation is based on the slightly higher degree of sequence homology to *E. coli spoT* than to *relA*. A functional analysis was carried out for the *relA* genes of *S. coelicolor* A3(2) and *B. subtilis*, which have been shown to encode enzymes with (p)ppGpp synthetase activity (Chakraborty *et al.*, 1996; Wendrich & Marahiel, 1997). (p)ppGpp accumulation assays in *C. glutamicum* wild-type and *rel* mutant strains, which lack a central 400 bp fragment (corresponding to amino acid residues 129–266), established that the *rel* gene encodes a (p)ppGpp synthetase. Synthesis of (p)ppGpp invoked by amino acid starvation was abolished in the *rel* mutants but not in the wild-type strains, a phenotype described for *relA* mutants of *E. coli* (Cashel *et al.*, 1996). As a result, further attempts to isolate a functional orthologue of the *E. coli spoT* gene were performed. However, heterologous hybridization experiments failed to identify a second *relA/spoT* homologue in *C. glutamicum*. Thus, it appeared possible that the *rel* gene encodes the functions residing in two *E. coli* genes. This thesis is supported by the following findings in several other organisms. (i) Data obtained from genome sequencing projects of *Mycobacterium leprae* and *Mycobacterium tuberculosis* (URL <http://kiev.physchem.kth.se/MycDB.html>), *Streptococcus pyogenes* (URL <http://dna1.chem.uoknor.edu>), *Helicobacter pylori* (Tomb *et al.*, 1997), *Mycoplasma genitalium* (Fraser *et al.*, 1995), *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996) and *Bacillus subtilis* (URL

<http://138.102.88.140>) reveal the appearance of only one *rel* equivalent in each of these bacteria. (ii) A chromosomal deletion in the *relA* gene of *B. subtilis* (Wendrich & Marahiel, 1997) has been shown to inactivate (p)ppGpp accumulation upon amino acid deprivation, as in *E. coli relA* mutants, as well as upon glucose exhaustion, a phenotype caused by certain *spoT* mutations in *E. coli* (Gentry & Cashel, 1996). (iii) The *S. equisimilis* H46A *rel* gene, like *E. coli spoT*, is bi-functional, encoding a strong (p)ppGppase and a weaker ribosome-independent (p)ppGpp synthetase. However, a *rel* insertion mutant fails to accumulate (p)ppGpp following amino acid starvation (Mechold *et al.*, 1996), a behaviour characteristic of *E. coli relA* but not *spoT* mutants.

In *C. glutamicum* an additional hint came from *rel* expression studies in *E. coli*, also giving evidence for a (p)ppGpp-degrading activity of the Rel protein. Growth inhibition, caused by overexpression of the *E. coli relA* gene, leading to increasing amounts of (p)ppGpp, could be reversed by the *C. glutamicum rel* gene. There may be other reasons for this behaviour; for example the *E. coli relA* gene product could be inactivated by the *C. glutamicum* Rel protein or *rel* gene expression might inhibit IPTG induction of active RelA protein. However, we strongly favour our conclusion, since similar experiments with the *rel* gene of *S. equisimilis* have shown almost identical results and the (p)ppGppase activity of the Rel protein deduced from these experiments could be verified *in vitro* (Mechold *et al.*, 1996).

Taken together, the data presented in this study make it likely that the *rel* gene of *C. glutamicum* is bifunctional, encoding a (p)ppGpp synthetase as well as a (p)ppGpp-degrading enzyme activity. The results also lead to the proposal that the metabolism of (p)ppGpp in *C. glutamicum* is mainly mediated by a single *rel* gene, which fulfils the functions that reside in the *relA* and *spoT* genes of *E. coli*. In addition, recent findings in *C. glutamicum* have indicated that RNA synthesis and growth rate are inversely correlated with the cellular ppGpp concentration (Ruklisha *et al.*, 1995), supporting an essential role of the *rel* gene in the stringent response of *C. glutamicum*.

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