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Cloning and Characterization of a DNA Region Encoding a Stress-Sensitive Restriction System from *Corynebacterium glutamicum* ATCC 13032 and Analysis of Its Role in Intergeneric Conjugation with *Escherichia coli*

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RP4-mediated transfer of mobilizable plasmids in intergeneric conjugation of *Escherichia coli* donors with *Corynebacterium glutamicum* ATCC 13032 is severely affected by a restriction system in the recipient that can be inactivated by a variety of exogenous stress factors. In this study a rapid test procedure based on intergeneric conjugal plasmid transfer that permitted the distinction between restriction-negative and restriction-positive *C. glutamicum* clones was developed. By using this procedure, clones of the restriction-deficient mutant strain *C. glutamicum* RM3 harboring a plasmid library of the wild-type chromosome were checked for their restriction properties. A complemented clone with a restriction-positive phenotype was isolated and found to contain a plasmid with a 7-kb insertion originating from the wild-type chromosome. This plasmid, termed pRES806, is able to complement the restriction-deficient phenotype of different *C. glutamicum* mutants. Sequence analysis revealed the presence of two open reading frames (*orf1* and *orf2*) on the complementing DNA fragment. The region comprising *orf1* and *orf2* displayed a strikingly low G+C content and was present exclusively in *C. glutamicum* strains. Gene disruption experiments with the wild type proved that *orf1* is essential for complementation, but inactivation of *orf2* also resulted in a small but significant increase in fertility. These results were confirmed by infection assays with the bacteriophage CL31 from *Corynebacterium lilium* ATCC 15990.

Recent studies have shown that there are no fundamental barriers preventing conjugal transfer of genetic information between phylogenetically distant species (see references 11, 13, 16, and 26 for recent reviews). The mobilizations of plasmids between gram-negative and gram-positive bacteria (27, 37, 47, 48, 50), from bacteria to yeasts (17, 42), and from bacteria to plants (8) are examples of conjugation between what were formerly thought to be incompatible partners. Therefore, the continuation of distinct species in nature seems to be mainly supported by mechanisms directed against the establishment of foreign DNA in the cell. In microorganisms, one of these barriers may be due to restriction enzymes which have been found in a wide range of gram-negative and gram-positive bacteria (see reference 32 for an overview). However, the role of restriction enzymes in intergeneric conjugation so far has been poorly investigated.

In previous work, we have shown that mobilizable shuttle plasmids can be transferred from gram-negative *Escherichia coli* to several gram-positive coryneform bacteria by conjugation (37). In these mating experiments, we used the *E. coli* donor strain S17-1 (43), which contains a derivative of the conjugative *Inc*P plasmid RP4 (10) integrated into the chromosome, and shuttle plasmids carrying selectable markers and the RP4 origin of transfer (*oriT*). RP4-driven mobilization of the shuttle plasmids was increased after introduction of a heat treatment step of the coryneform recipient cells prior to mating, or, alternatively, by using a restriction-deficient recipient strain. This led us to the presumption that the heat treatment step might cause an inactivation of the restriction system of the recipient. In a subsequent study (36), we were able to identify a whole set of different stress factors, including heat, ethanol, acids, bases, and sodium dodecyl sulfate, that are able to induce a conjugal competence in *Corynebacterium glutamicum* ATCC 13032. We could exclude that exogenous stress facilitates interspecific transfer in the course of a complex stress response in the recipient cells. Using infection assays conducted on *C. glutamicum* with bacteriophage CL31 (55) grown on *Corynebacterium lilium* ATCC 15990, we showed in vivo that the stress factors mentioned above directly impair the ability of *C. glutamicum* to restrict foreign DNA.

There is only a little information about restriction systems in coryneform bacteria. To our knowledge, none has so far been described for strains of the *C. glutamicum-Brevibacterium ammoniagenes* taxonomic cluster (41), although these strains are of industrial importance in the fermentative production of amino acids. In the work presented here, we describe the isolation and characterization of a DNA region responsible for the main restriction system in *C. glutamicum*. This restriction system is stress sensitive and active on DNA transferred by RP4-mediated conjugation.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and growth conditions. All bacterial strains, plasmids, and the bacteriophage used are listed in Table 1. *E. coli* and coryneform strains were grown in Luria-Bertani (LB) medium (33) at 37 and 30°C, respectively. When needed, kanamycin or chloramphenicol was added to final concentrations of 50 μg/ml to *E. coli* cultures and of 25 and 10 μg/ml, respectively, to *C. glutamicum* cultures. For infection assays with the bacteriophage CL31, coryneform

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TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this work

Strain, plasmid, or phage	Relevant characteristics	Source or reference[a]
Strains		
E. coli S17-1	*hsdR pro recA* carrying RP4-2-Tc::Mu in the chromosome	43
E. coli DH5α	F⁻ *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	14
Arthrobacter albidus DSM 20128	Wild-type strain	DSM
C. glutamicum ATCC 13058	Glutamic acid-producing strain	ATCC
C. glutamicum ATCC 13032	Wild-type strain	ATCC
C. glutamicum RM3	Restriction-deficient mutant of ATCC 13032	37
C. glutamicum RM4	Restriction-deficient mutant of ATCC 13032	36
C. glutamicum AS019	Spontaneous Rif^r mutant of ATCC 13059	56
C. glutamicum R127	Restriction-deficient mutant of AS019	23
C. glutamicum R167	Restriction-deficient mutant of AS019	23
C. glutamicum 806	= RM3(pRES806)	This work
C. glutamicum ASC9	ATCC 13032 with pASC9 integrated into the chromosome	This work
C. glutamicum ASC10	ATCC 13032 with pASC10 integrated into the chromosome	This work
C. lilium ATCC 15990	Wild-type strain	55
B. lactofermentum ATCC 13869	Wild-type strain	ATCC
B. flavum ATCC 14067	Wild-type strain	ATCC
B. ammoniagenes DSM 20305	Wild-type strain	DSM
B. stationis DSM 20302	Wild-type strain	DSM
B. divaricatum DSM 20297	Wild-type strain	DSM
Plasmids		
pCV22	*C. glutamicum* cloning vector; Km^r	40
pK18mob	Mobilizable sequencing vector; Km^r	38
pECM2	Mobilizable *E. coli-C. glutamicum* shuttle plasmid; Km^r Cm^r	21
pEBM3	Mobilizable *E. coli-C. glutamicum* shuttle plasmid; Km^r Cm^r	This work
pRES806	pCV22 with 7-kb chromosomal insert complementing *C. glutamicum* RM3	This work
pRES807	Deletion derivative of pRES806 that lacks the 0.8-kb *Xba*I fragment	This work
pRES808	Deletion derivative of pRES806 that lacks the 1.5-kb *Mlu*I fragment	This work
pRES809	Deletion derivative of pRES806 that lacks the 3-kb *Hpa*I-*Sma*I fragment	This work
pASC9	pK18mob carrying an internal 350-bp *Bgl*II-*Eco*RI fragment of *orf1*	This work
pASC10	pK18mob carrying an internal 290-bp fragment of *orf2* downstream of *Hin*dIII	This work
Phage CL31	Bacteriophage of *C. lilium* ATCC 15990	55

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany.

strains were grown in LB medium in the presence of 10 mM $CaCl_2$.

DNA manipulations. Plasmid DNA from *E. coli* cells was isolated by the method of Holmes and Quigley (19). Plasmid DNA was extracted from *C. glutamicum* by a slightly modified alkaline lysis method (5). Total chromosomal DNA of *C. glutamicum* and other coryneform strains was prepared by the procedure of Altenbuchner and Cullum (2).

DNA restriction, agarose gel electrophoresis, the filling in of blunt ends with Klenow polymerase, treatment with alkaline phosphatase, and ligation were carried out as described by Sambrook et al. (33). All enzymes for DNA manipulations were purchased from Pharmacia (Freiburg, Germany). DNA restriction fragments were isolated from agarose gels by using the USB Bioclean MP Kit delivered by United States Biochemicals (Bad Homburg, Germany), according to the manufacturer's recommendations.

Hybridization experiments. After DNA gel electrophoresis, Southern blots (45) were prepared on nylon membranes (Hybond-N; Amersham, Braunschweig, Germany) using a vacuum blotter (VacuGene; Pharmacia, Freiburg, Germany). Immobilization of DNA, labeling of DNA probes, and hybridization were performed with the DNA Labeling and Detection Kit—Nonradioactive from Boehringer (Mannheim, Germany), according to the manufacturer's specifications.

Bacterial transformation, electroporation, and conjugal plasmid transfer. *E. coli* was transformed by the RbCl method (14) or by electroporation with the GS Gene Pulser apparatus (Bio-Rad, Munich, Germany). *E. coli* was prepared for electroporation according to the recommendations of the Bio-Rad manual. For electroporation of *C. glutamicum* RM3, cells were grown at 30°C in LB medium containing 2.5% glycine, 0.1% Tween 80, and 1.5 mg of isonicotinic acid hydrazide (INH) per ml in an air incubator at 200 rpm and were harvested at an optical density at 580 nm of 0.4. For *C. glutamicum* ATCC 13032, 2.5 mg of INH per ml was added. Further steps were carried out essentially as described by Haynes and Britz (15). Intergeneric conjugal transfer of plasmids from *E. coli* to coryneform bacteria was performed as described by Schäfer et al. (37), with minor modifications as mentioned by Schwarzer and Pühler (39). The numbers of transconjugants presented in this study are results of matings of 4×10^8 donor cells with an equal number of recipients. Transconjugants were selected on LB agar containing 50 μg of nalidixic acid per ml and either 25 μg of kanamycin per ml or 10 μg of chloramphenicol per ml.

Construction of a genomic library of the *C. glutamicum* wild type. Chromosomal DNA was isolated from *C. glutamicum* ATCC 13032 and cut partially with *Sau*3A restriction endonuclease. Size fractionation of the DNA was done by electrophoresis in a 0.5% agarose gel with *Pst*I-cut λ-DNA as a size standard. DNA fragments of 5 to 10 kb were recovered from the agarose gel and purified with the USB Bioclean MP Kit. Subsequently, the fragments were inserted into *Bam*HI-cleaved and dephosphorylated plasmid pCV22 (40). The ligation mixture was electroporated into *C. glutamicum* RM3.

Curing of plasmids in *C. glutamicum*. Plasmid-free strains were generated as follows: nonselective LB medium was inoculated with a single colony of the plasmid-containing strain and grown to an optical density at 580 nm of 0.2 at 30°C. Subsequently, the cells were incubated for 24 to 48 h at 41.5 to 42°C with permanent shaking at 120 rpm. The cells were then plated onto nonselective LB agar and incubated for 48 h at 30°C. Colonies were then checked for plasmid-encoded antibiotic resistance and for their plasmid contents by lysis.

DNA sequencing and sequence analysis. After subcloning of fragments into plasmid pK18mob (38), unidirectional deletions were introduced by using the Double Stranded Nested Deletion Kit (Pharmacia) which is based on the 3′-5′ exonuclease activity of exonuclease III. DNA sequencing analysis was performed with DNA isolated and purified with the Quiagen Plasmid Mini Kit (Diagen, Hilden, Germany) by the dideoxy chain termination method of Sanger et al. (34). The sequencing reactions were carried out with the AutoRead Sequencing Kit from Pharmacia. Electrophoretic analysis and detection of the sequencing products were accomplished with the help of an automated fluorescence DNA sequencer from Pharmacia (Piscataway, N.J.). Computer-assisted compilation of DNA sequences was done with the FASTA program package of Lipman and Pearson (25). Analysis of the DNA sequence, the search for open reading frames, and the analysis of the deduced amino acid sequences were carried out with the help of the ANALYSEQ, ANALYSEP, and DIAGON programs (46).

Preparation of phage lysate and infection assays. *C. lilium* ATCC 15990 cells were grown to reach logarithmic growth. The culture was then provided with a single plaque and incubated for 45 min at 37°C without shaking. A total of 0.2 ml of the infected culture was mixed with 3 ml of LB soft agar and poured onto prewarmed LB plates. After 24 h, plates showing confluent lysis were selected and 5 ml of SM buffer (10 mM NaCl, 10 mM $MgSO_4$, 20 mM Tris-HCl [pH 7.5]) was added. Subsequently, the plates were stored for 5 h at 4°C. The buffer was then recollected and subjected to centrifugation (10 min, 5,000 rpm, and 4°C). A total of 50 μl of chloroform was added to the supernatant, and the phage lysate (6.6×10^{10} PFU/ml) was stored at 4°C.

Infection of *C. glutamicum* strains in plate assays was performed as follows: *C. glutamicum* was grown in LB medium with 10 mM $CaCl_2$ to reach an optical density at 580 nm of approximately 0.4. A total of 0.25 ml of the culture was then mixed with 3 ml of LB soft agar and poured onto prewarmed solid LB medium. A total of 10 μl (each) of different dilutions of the phage lysate were dropped onto the bacterial lawn, and the agar plate was then incubated for 24 to 48 h at 37°C.

Nucleotide sequence accession number. The DNA sequence reported here is available in the GenBank under accession number U13922.

RESULTS

Construction of the mobilizable *E. coli-C. glutamicum* shuttle plasmid pEBM3. Plasmid pEBM3 (Fig. 1) is a shuttle plasmid that is stably maintained in *E. coli* as well as in *C. glutamicum*. It can be transferred by RP4-mediated conjugation and, in addition to the kanamycin resistance determinant, carries a chloramphenicol resistance gene which allows the selection for the plasmid in *C. glutamicum* with chloramphenicol concentrations of up to 50 μg/ml.

In order to construct plasmid pEBM3, the mobilizable *E. coli* plasmid pK18mob (38) was partially cleaved with the restriction endonuclease *Pvu*II and religated to remove a 630-bp *Pvu*II fragment comprising the multiple cloning site

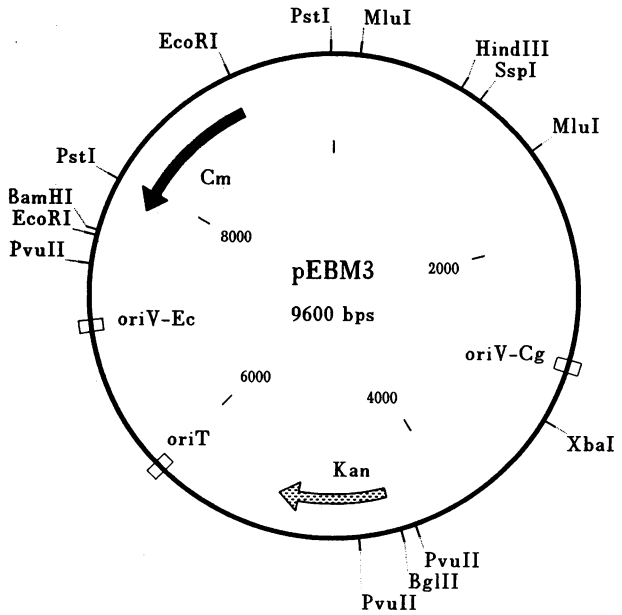

FIG. 1. Mobilizable *E. coli-C. glutamicum* shuttle plasmid pEBM3 used for plate mating assays. Abbreviations: oriV-Ec, origin for vegetative replication in *E. coli*; oriV-Cg, origin for vegetative replication in *C. glutamicum*; oriT, origin for transfer replication; Kan, kanamycin resistance gene; Cm, chloramphenicol resistance gene from plasmid pTP10 (22). For details of construction, see text.

and the *lacZ*α fragment. This procedure generated the deletion derivative pEM11 which was cleaved partially with *Pvu*II. A 2.3-kb *Pvu*II fragment carrying the chloramphenicol resistance gene originating from the R plasmid pTP10 (22) from *Corynebacterium xerosis* was subsequently inserted into the linearized plasmid pEM11 to give plasmid pEM12. In order to obtain a plasmid which is able to replicate in coryneform strains, plasmid pEM12 was treated with *Hin*cII and ligated to the 4.2-kb *Sca*I fragment of plasmid pBL1, a cryptic plasmid from *B. lactofermentum* (35).

Complementation of the restriction-deficient mutant *C. glutamicum* RM3. The cloning of genes encoding restriction endonucleases in a foreign host, e.g., *E. coli*, may be problematic. Even if the gene encoding the methyltransferase (*hsdM*) is cloned along with the gene for restriction (*hsdR*), the chromosome of the new host may be exposed to an active restriction endonuclease before the target sequences are entirely masked by modification. Therefore, most restriction-modification systems have been cloned stepwise with the gene for the methylase first (53, 54). In order to clone the gene(s) responsible for restriction in *C. glutamicum*, we have applied a different strategy. We used complementation of the restriction-minus mutant *C. glutamicum* RM3 (37) with *C. glutamicum* wild-type DNA, combined with a procedure to identify complemented clones that is based on interspecific conjugal plasmid transfer. For this screening method, we took advantage of the observation that matings between *E. coli* and *C. glutamicum* are rather inefficient when conducted on agar surfaces instead of nitrocellulose filters. The low efficiency most probably is due to the rigid nature of P-type pili specified by plasmid RP4 (7). A genomic library of *C. glutamicum* ATCC 13032 was established in plasmid pCV22 (40), a cloning vector based on the replicon pHM1519 (28), and introduced into *C. glutamicum* RM3 by electroporation. The resulting clones were checked for their restriction properties in plate mating assays with *E. coli* S17-1

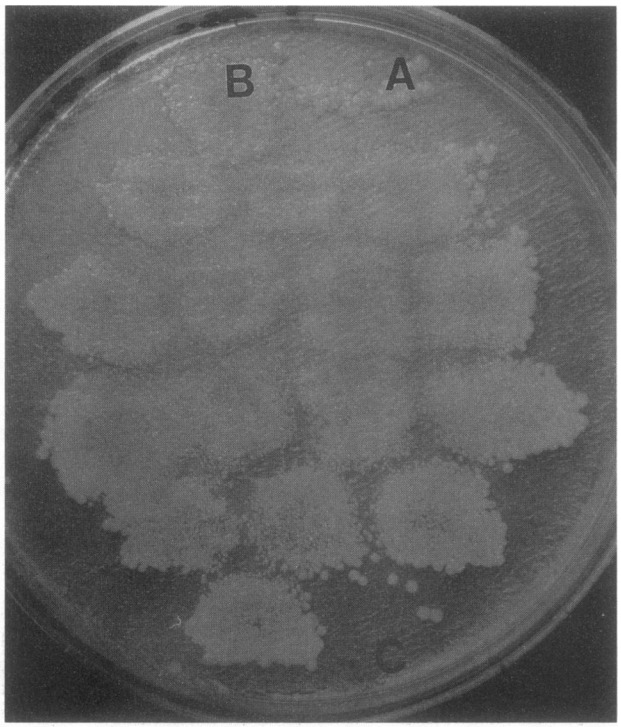

FIG. 2. Identification of a *C. glutamicum* RM3 clone, containing a plasmid which confers low conjugation efficiency by a plate mating assay (see Materials and Methods for experimental details). *E. coli* S17-1(pEBM3) donor cells were mated with *C. glutamicum* RM3 clones harboring a genomic library of the wild type in plasmid pCV22. Results are shown from matings with 16 *C. glutamicum* clones containing plasmids with different genomic inserts and from matings with the wild type (A) and RM3 (B) as controls. The *C. glutamicum* clone 806 (C) showing a low fertility in interspecific matings was isolated. This clone carries a plasmid that complements the restriction deficiency phenotype of *C. glutamicum* RM3.

containing pEBM3 as the donor. Plasmid pEBM3 is compatible with pCV22 derivatives, and its presence in transconjugants can be selected for by chloramphenicol. For plate mating assays, 150 μl of a stationary-phase donor culture of *E. coli* S17-1(pEBM3) was plated on LB agar prewarmed to 37°C. Recipient clones were applied to the donor by using sterile pipettes, and the plate was incubated for 20 h at 30°C. Subsequently, the mating assay was replica plated onto selective LB medium containing chloramphenicol and nalidixic acid. The number of transconjugants can be estimated after 48 h of incubation at 30°C. When the mating was conducted with a restriction-positive clone, no or only a few transconjugants were observed, whereas many transconjugants harboring pEBM3 in addition to the pCV22 derivative arose from matings with a restriction-deficient clone. Therefore, this test allows one to discriminate clearly between restriction-negative and restriction-positive clones. All of the *C. glutamicum* RM3 clones harboring the genomic library in pCV22 were used as recipient clones in plate mating assays, with the *C. glutamicum* wild-type and RM3 strains serving as control recipients (Fig. 2). One of 2,200 tested clones, *C. glutamicum* 806, displayed a restriction-positive phenotype, yielding a small number of transconjugants (Fig. 2).

Analysis of the plasmid pRES806, which complements the restriction-deficient mutant *C. glutamicum* RM3. *C. glutamicum* 806 was found to contain the 11.4-kb plasmid pRES806 that is responsible for the restriction-positive phenotype. Upon retransformation of the plasmid into *C. glutamicum* RM3, all transformants tested were restriction positive as revealed by plate mating assays. Plasmid-free cells obtained by temperature curing again showed a restriction deficiency phenotype.

TABLE 2. Titers of transconjugants in matings of *E. coli* S17-1(pEBM3) with *C. glutamicum* RM3 or ATCC 13032[a]

Recipient	No. of transconjugants	
	Without heat treatment	With heat treatment
RM3	$5 \times 10^7 \pm 1.5 \times 10^7$	$1 \times 10^7 \pm 1.5 \times 10^7$
RM3(pRES806)	$6 \times 10^3 \pm 3 \times 10^3$	$2 \times 10^5 \pm 4 \times 10^5$
ATCC 13032	$3 \times 10^3 \pm 3 \times 10^3$	$2 \times 10^7 \pm 2.5 \times 10^7$
ATCC 13032(pRES806)	0	$4 \times 10^3 \pm 5 \times 10^3$

[a] Matings were done in the presence or absence of plasmid pRES806, with and without a preceding heat treatment (for 9 min and at 48.5°C) of the recipient. Means of the numbers of transconjugants from three independent matings, with standard deviations, are listed.

The plasmid pRES806 was introduced into *C. glutamicum* wild-type cells by electroporation, and filter matings were performed with RM3 and the wild type in the absence and presence of pRES806, with *E. coli* S17-1(pEBM3) as the donor. The results (Table 2) indicate that the presence of pRES806 in the recipient reduced the fertility by 4 orders of magnitude. RM3 cells harboring pRES806 allowed transfer efficiencies comparable to those obtained with wild-type cells, whereas no transconjugants at all could be observed with wild-type cells carrying pRES806. Heat treatment of strain RM3(pRES806) prior to mating resulted in a significant increase in the number of transconjugants. However, the increase is smaller compared with the heat-induced increase obtained with plasmid-free wild-type cells. This may be due to the fact that the complementing plasmid is present in several copies per cell. When pRES806 was present in wild-type cells, we obtained transconjugants exclusively after heat treatment.

As shown in Table 3, plasmid pRES806 is able to complement several restriction-deficient mutants derived by chemical mutagenesis of *C. glutamicum* ATCC 13032 (37) or from *C. glutamicum* AS019 (23). It should be mentioned that we were not able to introduce plasmid pRES806 by electroporation into *B. lactofermentum* ATCC 13869, a strain which is very closely related to *C. glutamicum*.

A restriction endonuclease recognizes and degrades foreign DNA. DNA which carries the species-specific methylation

TABLE 3. Plasmid pRES806 is able to complement different restriction-deficient mutants of *C. glutamicum* strains ATCC 13032 and AS019

Recipient	Endogenous plasmid	No. of transconjugants[a]
ATCC 13032		4×10^3
RM3		5×10^7
RM3	pCV22	3×10^7
RM3	pRES806	7×10^3
RM4		5×10^6
RM4	pRES806	2×10^2
AS019		4×10^2
R127		3×10^7
R127	pRES806	1×10^3
R163		7×10^6
R163	pRES806	5×10^1

[a] Shown are the means of the total numbers of transconjugants from three independent matings with *E. coli* S17-1(pECM2) as the donor strain.

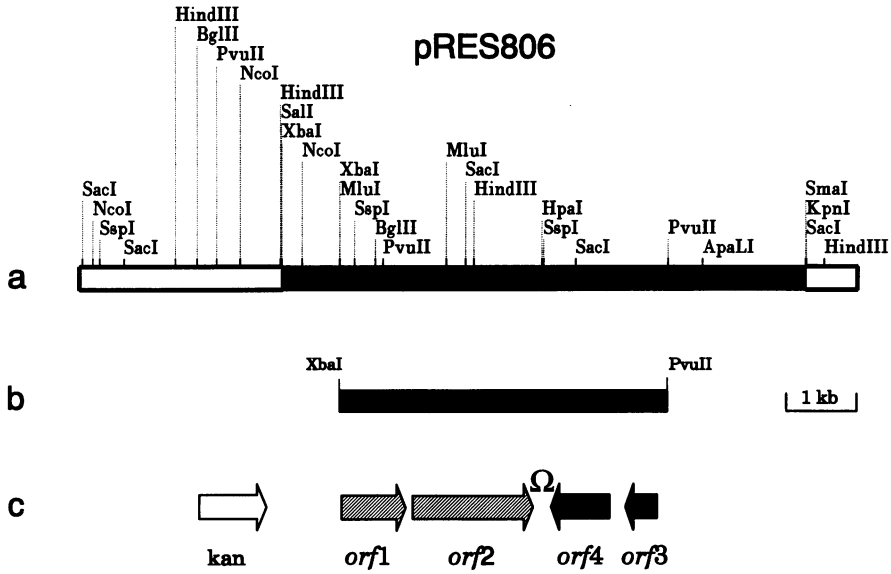

FIG. 3. (a) Detailed restriction map of plasmid pRES806 and organization of the DNA fragment containing *orf1* and *orf2*. The 7-kb chromosomal insertion of plasmid pRES806 that complements the restriction deficiency of *C. glutamicum* RM3 is presented in black. Vector sequences are depicted as an open box (see panel c). (b) The sequenced *Xba*I-*Pvu*II fragment is rendered prominent. (c) The identified *orf1* and *orf2* in the DNA region essential for complementation are shown as hatched arrows. *orf3* and *orf4*, which are dispensable for complementation, are shown as shaded arrows. The location of the kanamycin resistance gene is given as an open arrow. Ω, DNA sequence that might function as a rho-independent terminator of transcription.

pattern is protected against degradation by the corresponding endonuclease. To demonstrate that pRES806 encodes a restriction activity from *C. glutamicum* ATCC 13032, we isolated DNA of the plasmid pEBM3 from *E. coli* S17-1 and from *C. glutamicum* ATCC 13032 cells. The DNA concentrations were adjusted to 1.8 μg/ml each, and *C. glutamicum* RM3 and RM3(pRES806) cells were electroporated with both plasmid preparations. Electroporation of strain RM3 worked equally well with homologous DNA isolated from *C. glutamicum* (5×10^5 transformants) and with heterologous DNA isolated from *E. coli* S17-1 (9×10^4 transformants). RM3(pRES806) cells were susceptible to homologous pEBM3 DNA (8×10^5 transformants) but were poorly transformed by heterologous pEBM3 DNA (2×10^2 transformants). From these results, it can be concluded that plasmid pRES806 encodes a function that specifically impairs the introduction of heterologous DNA.

Plasmid pRES806 is composed of the vector plasmid pCV22 used for construction of the genomic library and of a 7-kb DNA insert originating from the chromosome of the *C. glutamicum* wild type. A detailed restriction map of the plasmid is shown in Fig. 3a.

Sequence determination and analysis of a DNA subfragment complementing the restriction deficiency of *C. glutamicum* RM3. We have determined the entire DNA sequence of the 4.85-kb *Xba*I-*Pvu*II subfragment of plasmid pRES806 complementing the restriction deficiency of *C. glutamicum* RM3 (Fig. 3b). The organization of the sequenced DNA region is depicted in Fig. 3c. Four open reading frames (*orf1* to *orf4*) could be identified by coding region analysis. Deletion analyses demonstrated that the DNA region comprising *orf1* and *orf2* is sufficient for complementation of the mutant strain RM3: deletion derivative pRES807 was obtained by deleting from plasmid pRES806 the 0.8-kb *Xba*I fragment upstream of *orf1* (Fig. 3a). Mating experiments with RM3 harboring pRES807 (Table 4) showed that this plasmid is able to complement the restriction deficiency of RM3. A 1.5-kb *Mlu*I fragment comprising *orf1* and the proximal part of *orf2* (Fig. 3a) was deleted to give the derivative pRES808. This plasmid has completely lost the ability to complement the restriction deficiency of RM3. Finally, we generated plasmid pRES809 by deleting the 3-kb *Hpa*I-*Sma*I fragment (Fig. 3a), thereby removing the last 108 bp from *orf2* and the sequence downstream of *orf2*. As shown in Table 4, pRES809 has lost a small part of its complementing activity. These data therefore revealed that *orf1* encodes the major restriction activity, that *orf2* contributes to a small but significant extent to complementation, and that there are no other genes involved in restriction on plasmid pRES806. The DNA sequence of the part comprising *orf1* and *orf2*, along with the deduced amino acid sequence, is listed in Fig. 4.

Translation of *orf1* starts at an ATG codon (nucleotides [nt] 88 to 90) or at a GTG codon (nt 109 to 111). We were not able to identify an obvious ribosome-binding site as judged by complementarity to the 3′ end of 16S rRNA of *Bacillus subtilis* (30) upstream of these prospective translational starts. Translation of *orf1* stops at a TAA codon which is located at nt 1162 to 1164. If translation initiated at the first ATG codon, *orf1* would comprise 1,074 nt encoding a 358-amino-acid, 39.8-kDa protein.

TABLE 4. Results from mating experiments using *E. coli* S17-1(pEBM3) as the donor with various recipient strains

Recipient	Intact *orf1*	Intact *orf2*	No. of transconjugants[a]
RM3	−	−	$4 \times 10^7 \pm 2.5 \times 10^7$
RM3(pRES806)	+	+	$5 \times 10^3 \pm 2.5 \times 10^3$
RM3(pRES807)	+	+	$3.5 \times 10^3 \pm 2.4 \times 10^3$
RM3(pRES808)	−	−	$3 \times 10^7 \pm 1.5 \times 10^7$
RM3(pRES809)	+	−	$2 \times 10^4 \pm 1 \times 10^4$

[a] Means of the numbers of transconjugants from three independent matings, with standard deviations, are given.

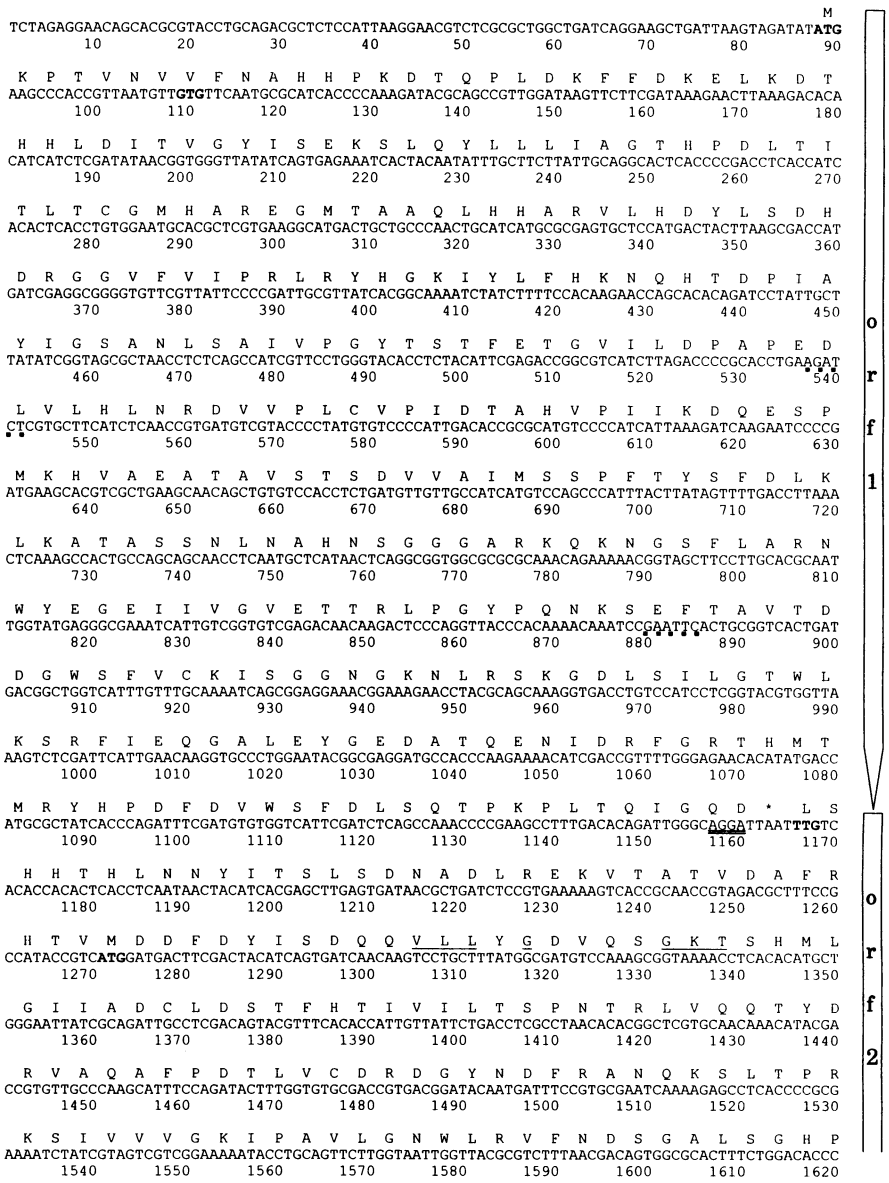

FIG. 4. Nucleotide sequence of the DNA region comprising *orf1* and *orf2*. Possible translational start codons are in boldface type, and stop codons are marked by asterisks. A putative ribosomal binding site upstream of *orf2* is marked by a double line. The motifs A and B, which may be involved in NTP binding, are underlined. The inverse repetitive sequence downstream of *orf2* that might act as a bidirectional terminator of transcription is shaded. Relevant restriction sites are marked by dotted lines. The 350-bp fragment used for disruption of *orf1* extends from the *Eco*RI site at nt 880 to 885 to the *Bgl*II site at nt 537 to 542. The DNA fragment used to disrupt *orf2* was cloned from a deletion derivative obtained by exonuclease III treatment and comprises 290 bp upstream of the *Hin*dIII site at nt 1987 to 1992.

There are two possible translational starts for *orf2*. Translation could start at an ATG codon (nt 1271 to 1273) which lacks a canonical ribosome-binding site. In this case *orf2* would encode a protein of 597 amino acids and 66.8 kDa. However, translation probably initiates at a TTG codon (nt 1166 to 1168), since it is preceded by a possible RBS (-AGGA-; nt 1157 to 1160), and the coding probability increases immediately at this codon (not shown). TTG codons are infrequently used as translational start codons in some gram-positive bacteria (30). Translation of *orf2* ends at a TAA codon (nt 3062 to 3064). The use of the TTG codon as a translational start site would result in a protein of 632 amino acids and 70.8 kDa.

The use of the first ATG codon as the translational start would generate an intergenic region of 109 bp between *orf1* and *orf2*. Since we could not detect any rho-independent terminator-like sequences in this DNA region, we presume that *orf1* and *orf2* might constitute an operon. We found no DNA sequence resembling the −35 and −10 regions of typical *E. coli* promoters in the 88 bp upstream from *orf1* sequenced so far. However, we identified a G+C-rich, inverted repeat downstream from *orf2* (nt 3090 to 3125) which is followed by a series of T residues (Fig. 4). The structure shows a striking similarity with typical bidirectional, rho-independent terminators of *E. coli* (1). It is likely that transcription of *orf1* and *orf2* is terminated at this structure.

Two other open reading frames (*orf3* and *orf4*) have been

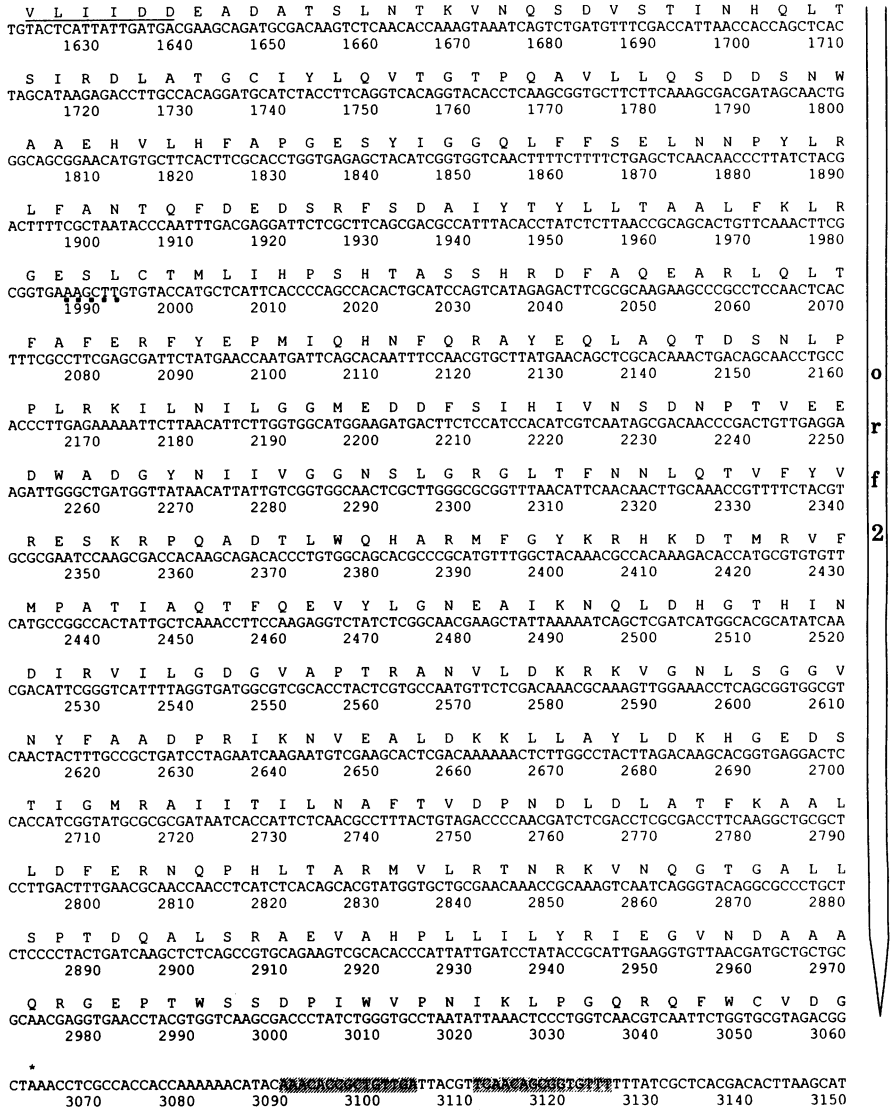

FIG. 4—*Continued.*

detected on the opposite strand. They are dispensible for complementation, and their analysis will be part of another study. The average G+C ratio of *orf1* to *orf2* is about 49% which is remarkably low since *C. glutamicum* genes sequenced so far display an average G+C content of 55 to 56%. The DNA sequences of *orf1* and *orf2* as well as the deduced amino acid sequences were compared with sequences stored in the actual versions of the EMBL and GenBank nucleotide sequence databases and the NBRF and SwissProt protein databases. There is no striking homology to any nucleotide or amino acid sequence stored in these databases. However, *orf2* shows the A and B amino acid motifs that are conserved in NTP-binding domains of various proteins (52): The sequence **V-L-L-Y-G-D-V-Q-S-G-K-T-** (Fig. 4, nt 1304 to 1339) strongly resembles the A motif (Walker motif). The sequence **-V-L-I-I-D-D-** (Fig. 4, nt 1622 to 1639) probably constitutes the B motif.

Southern blot experiments were performed to detect DNA regions homologous to *orf1* and *orf2* in other coryneform bacteria. For this purpose, the 2.9-kb *Xba*I-*Hpa*I DNA fragment of the chromosomal insert in plasmid pRES806 comprising the entire *orf1* and the 5′ part of *orf2* (Fig. 3a) was isolated and labeled with digoxigenin-dUTP. Total DNAs of *C. glutamicum* ATCC 13032, ATCC 13058, and AS019, *C. lilium* ATCC 15990, *Arthrobacter albidus* DSM 20128, *B. ammoniagenes* DSM 20305, *Brevibacterium divaricatum* DSM 20297, *Brevibacterium flavum* ATCC 14067, *B. lactofermentum* ATCC 13869, and *Brevibacterium stationis* DSM 20302 were isolated, digested with the restriction enzymes *Xba*I and *Hpa*I, and hybridized against the 2.9-kb *Xba*I-*Hpa*I fragment as a probe. Hybridization to a 2.9-kb chromosomal *Xba*I-*Hpa*I DNA fragment present exclusively in the three *C. glutamicum* strains was detected (data not shown). Although all the strains used for this experiment can be considered to be closely related and in fact—as is the case for *C. glutamicum* and *B. lactofermentum*—are sometimes regarded as one species (24), they differ in the DNA region comprising *orf1* and *orf2*. This DNA region therefore is unique to *C. glutamicum* and can be used to discriminate between *C. glutamicum* and closely related species.

TABLE 5. Number of transconjugants in matings of *E. coli* S17-1(pEBM3) with various *C. glutamicum* strains

Recipient	Relevant property	No. of transconjugants[a]
ATCC 13032	Wild type	$5 \times 10^3 \pm 4.1 \times 10^3$
ASC9	Disrupted *orf1*	$5 \times 10^6 \pm 2 \times 10^6$
ASC10	Disrupted *orf2*	$1 \times 10^4 \pm 3.5 \times 10^3$

[a] Means and standard deviations of the results of three independent matings are listed.

Genetic analysis of *orf1* and *orf2* by gene disruption experiments. In order to evaluate the biological function of *orf1* and *orf2*, we decided to disrupt both open reading frames in the chromosome of *C. glutamicum* ATCC 13032. For this purpose, an internal, 350-bp *Bgl*II-*Eco*RI fragment of *orf1* (Fig. 4) was isolated from plasmid pRES806 and cloned into the vector pK18mob cut with *Bam*HI and *Eco*RI. The recombinant plasmid pASC9 was subsequently introduced into the mobilizing strain *E. coli* S17-1 by electroporation.

An internal *Eco*RI-*Hin*dIII fragment of *orf2* covering 290 bp of *orf2* downstream of the *Hin*dIII-site (Fig. 4) was obtained from a subclone and inserted into pK18mob cut with *Eco*RI and *Hin*dIII to generate plasmid pASC10. Plasmid pASC10 also was transferred to *E. coli* S17-1, and both strains—*E. coli* S17-1 (pASC9) and *E. coli* S17-1 (pASC10)—were then used as donor strains in interspecific matings with heat-treated *C. glutamicum* wild-type cells. Neither plasmid pASC9 nor plasmid pASC10 is able to replicate in *C. glutamicum*. The plasmids therefore can only be stably maintained by integration into the chromosome via homologous recombination with the cloned internal fragments. Since these fragments do not comprise the 5′ and the 3′ regions of *orf1* and *orf2*, integration into the chromosome results in two incomplete copies of the respective open reading frame separated by the vector plasmid pK18mob. Transconjugants were selected, and correct integration was verified by Southern blot analysis. Integration of plasmid pASC9 led to the strain *C. glutamicum* ASC9 with a disrupted *orf1*. The disruption of *orf1* should also inactivate *orf2* if these open reading frames constitute an operon. Integration of plasmid pASC10 generated the strain *C. glutamicum* ASC10 which has a disrupted *orf2* but retains an intact *orf1*. The strains *C. glutamicum* ASC9 and ASC10 were used as recipients in intergeneric matings with *E. coli* S17-1(pEBM3) as the donor. Results of these matings are listed in Table 5. The disruption of *orf1* in the *C. glutamicum* wild type leads to a dramatic increase in fertility of about 3 orders of magnitude compared with that in wild-type recipients. Disruption of *orf2* caused slightly elevated transfer efficiencies. From these data, we conclude that *orf1* probably encodes the major restriction enzyme of *C. glutamicum*. Since the strain *C. glutamicum* ASC10 is viable, it is unlikely that *orf2* encodes the modification methylase pertinent to this restriction enzyme.

Functional analysis of *orf1* and *orf2*, using the bacteriophage CL31 from *C. lilium* ATCC 15990. Bacteriophages are classical tools to investigate restriction-modification systems in bacteria. The corynephage CL31 from *C. lilium* ATCC 15990 (55) was used to study the function of *orf1* and *orf2* in vivo. A lysate of CL31 (6.6×10^{10} PFU/ml) was prepared from its host strain *C. lilium* ATCC 15990. This lysate was used to infect *C. glutamicum* ATCC 13032, *C. glutamicum* RM3, *C. glutamicum* RM3 (pRES806), *C. glutamicum* ASC9, and *C. glutamicum* ASC10 cells in plate assays. Figure 5 shows that wild-type cells get poorly infected by CL31, whereas the restriction-deficient mutant *C. glutamicum* RM3 is susceptible to CL31 propagation. Plasmid pRES806 confers phage resistance to *C. glutamicum* RM3. Compared with the wild-type strain, *C. glutamicum* ASC9 and *C. glutamicum* ASC10 were infected by CL31 at a

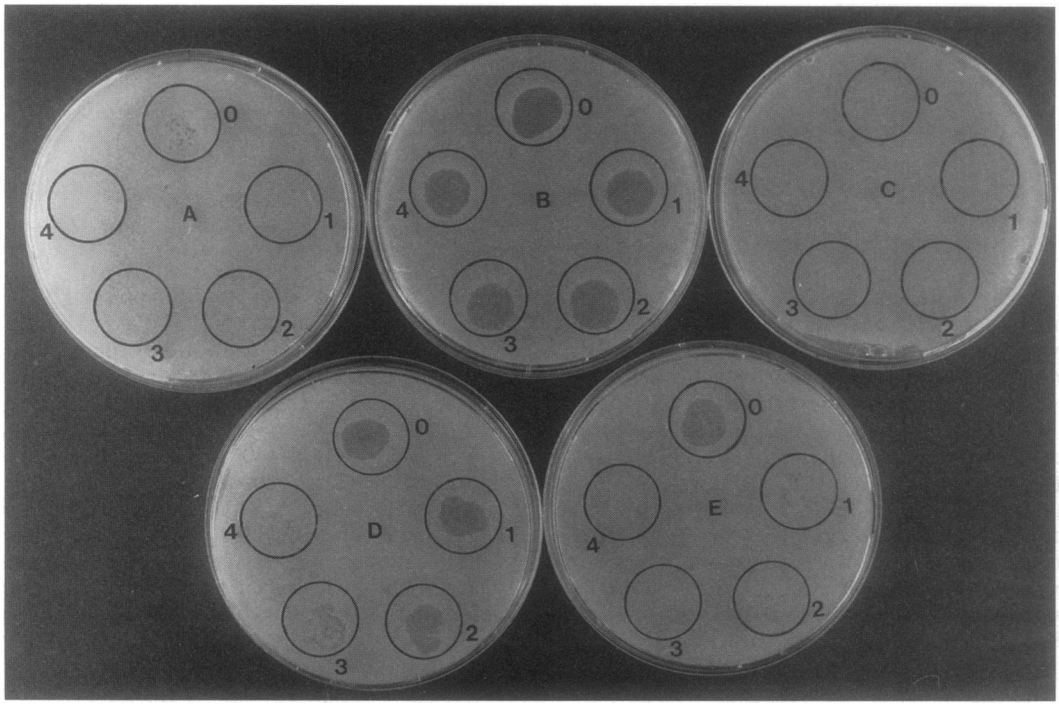

FIG. 5. Infection of the *C. glutamicum* strains ATCC 13032 (wild type) (A), RM3 (B), RM3(pRES806) (C), ASC9 (D), and ASC10 (E) with different dilutions (0 to −4) of a lysate (6.6×10^{10} PFU/ml) of bacteriophage CL31, which was prepared from *C. lilium* ATCC 15990. Details of the infection assay are mentioned in Materials and Methods.

highly and at a slightly increased frequency, respectively. All strains are lysed at a high frequency by phage CL31, if the lysate was prepared from *C. glutamicum* ATCC 13032 (data not shown). The results confirm that plasmid pRES806 encodes a restriction endonuclease from *C. glutamicum* ATCC 13032 that prevents propagation of phages grown on a different host strain. Using the same phage experiments, we could demonstrate in vivo that this restriction enzyme is sensitive to heat or ethanol, since wild-type cells previously exposed to stress are more sensitive to phage infection than untreated controls (data not shown).

DISCUSSION

In this study we describe the cloning and preliminary characterization of the main restriction system of *C. glutamicum* ATCC 13032. By complementation of the restriction-deficient mutant strain *C. glutamicum* RM3, and subsequent screening for restriction-positive clones using a rapid test procedure based on intergeneric conjugal transfer, we isolated plasmid pRES806 which carries a 7-kb insertion from the wild-type genome. From several points of view, it is obvious that plasmid pRES806 encodes a restriction enzyme from *C. glutamicum*: (i) when present in restriction-deficient mutants of *C. glutamicum*, or in wild-type cells, plasmid pRES806 reduces the fertility in intergeneric matings with *E. coli* by up to 4 orders of magnitude; (ii) plasmid pRES806 impairs the introduction of the vector pEBM3 into *C. glutamicum* RM3 by electroporation, if the pEBM3-DNA was isolated from *E. coli* cells, whereas it has no effect on transformation with homologous pEBM3-DNA; (iii) plasmid pRES806 could not be transferred into *B. lactofermentum* cells by electroporation, although the replicon—pHM1519 (28)—is known to function well in this strain; and (iv) plasmid pRES806 is able to protect RM3 cells against infection with the bacteriophage CL31, if the phage was grown on *C. lilium* ATCC 15990, but not if the phage was grown on *C. glutamicum* ATCC 13032.

Sequence analysis of a subfragment able to complement *C. glutamicum* RM3 revealed the presence of two open reading frames which probably constitute an operon. The sequenced DNA region displays a G+C content of about 49%, which is unusually low for *C. glutamicum* genes that normally have 55 to 56% G+C. For some unknown reason, G+C content that is atypically low for the species is pertinent to genes encoding *hsdR* and *hsdM* genes (4, 9), a fact which is still poorly understood.

It is striking that genes encoding restriction endonucleases usually share no significant homologies (53). Even the amino acid sequences of isoschizomeric enzymes often are completely different (53, 54). There is also no significant overall homology of *orf1* and *orf2* or the deduced amino acid sequences to any published gene or protein, respectively. However, we could detect amino acid sequences in *orf2* that resemble the conserved A and B motifs present in several proteins binding ATP, like ATPases (52). The A motif is characterized by a hydrophobic stretch of β-strand followed by the sequence -G/A-X-X-(G)-X-G-K-T-, while the B motif typically consists of a hydrophobic stretch of β-strand followed by -D-(D/E)-. Since *orf2* shows both motifs, we presume that *orf2* is an ATP-binding protein.

Southern blot experiments revealed that *orf1* and *orf2* are present in one copy per genome and that there are no similar DNA regions in strains which are closely related, or even in strains thought to be identical to *C. glutamicum*. Since it is known that coryneform strains harbor several prophage-like particles in their genomes (31, 44), it is conceivable that the species specificity of the DNA region is due to its location on a prophage the host range of which is restricted to *C. glutamicum* strains. It is known that prophages can carry genes for modification and restriction enzymes (20). Gene disruption experiments showed that inactivation of *orf1* resulted in a dramatic increase of fertility, whereas disruption of *orf2* only slightly raised fertility. We therefore conclude that *orf1*, which may encode a protein of 358 amino acids, is essential for complementation.

It is, however, possible that optimal restriction activity is generated by cooperation of the gene products encoded by *orf1* and *orf2*.

For the following reasons we presume that there exists a second, rather weak, restriction system in *C. glutamicum* wild-type cells that is also absent from RM3 cells: (i) complementation of RM3 with plasmid pRES806 leads only to wild-type fertility, although plasmid pRES806 is present in several copies per cell, and (ii) disruption of *orf1* in the wild-type chromosome (leading to *C. glutamicum* ASC9) resulted in a dramatic increase in the transfer efficiency, but fertility was still slightly lower than in *C. glutamicum* RM3.

There is no indication that pRES806 carries the *hsdM* gene along with the *hsdR* gene. Since *orf2* can be disrupted in the wild type without affecting the viability of the cells, *orf2* does not encode the corresponding methyltransferase.

The main *C. glutamicum* restriction system acts not only on foreign DNA that is transferred by phage infection or electroporation but also against DNA that enters the cell via RP4-mediated conjugation. This observation is noteworthy, because single-stranded DNA as it is transferred by conjugation usually is not a substrate for restriction in the recipient cells (6, 11). We presume that during synthesis of the complementary strand in the recipient, short patches of nonmethylated DNA that can be targeted by the restriction enzyme might occur. This hypothesis is consistent with the data from Trieu-Cuot and coworkers, who showed that the transfer of mobilizable plasmids from *E. coli* into *Bacillus subtilis* can be enhanced by removing recognition sites of the *Bsu*M restriction enzyme from the plasmids (49).

Restriction-modification systems in *C. glutamicum* and closely related strains of the *C. glutamicum-B. ammoniagenes* cluster have not been described so far. Vertes et al. have shown that *C. glutamicum* ATCC 31831 and *B. flavum* MJ233C harbor *mcr*-like restriction systems that recognize and degrade plasmid DNA that carries *E. coli*-specific methylations (51). However, if such a system existed in *C. glutamicum* ATCC 13032 and related strains, it did not act on DNA transferred by RP4-mediated conjugation, as judged by conjugation experiments with *dam dcm*-proficient and -deficient *E. coli* donor strains (data not shown). There is therefore no requirement for nonmethylated DNA in interspecific conjugation experiments.

A striking feature of the restriction system of *C. glutamicum* is its stress sensitivity. Fertility in intergeneric matings and sensitivity to phage infection can be induced by several exogenous stress conditions, and this makes interspecific conjugation the most efficient way to introduce DNA from *E. coli* into *C. glutamicum* and related species. The stress sensitivity of the restriction system of *C. glutamicum* might have the following reasons: (i) restriction enzymes in *C. glutamicum* may be generally more unstable than other cellular components, and (ii) the enzymes might be located at or near to the cell surface, where they are more directly exposed to the stress effectors.

Since heat sensitivity of restriction has been reported for other bacteria (3, 12, 18, 29), stress sensitivity might be a more general property of restriction enzymes. Under stress, restriction would be alleviated and the cell could acquire foreign

genetic information more easily, and that might enhance the capability to deal with the particular environmental requirement.

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