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Construction of a Lactococcal Expression Vector: Expression of Hen Egg White Lysozyme in *Lactococcus lactis* subsp. *lactis*

MAARTEN VAN DE GUCHTE,* JOS M. B. M. VAN DER VOSSEN, JAN KOK, AND GERARD VENEMA

Department of Genetics, Centre of Biological Sciences, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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A pair of vectors for expression of heterologous genes in *Lactococcus lactis* was constructed. In addition to an origin of replication that has a broad host range, these vectors contain a multiple cloning site flanked by gene expression signals originating from *L. lactis* subsp. *cremoris* Wg2. The two vectors, about 3.7 kilobase pairs in size, differ only in the type of antibiotic resistance they confer to their hosts. pMG36 carries a kanamycin resistance marker, which was replaced by an erythromycin resistance marker in pMG36e. As an example of the use of these vectors, the hen egg white lysozyme-coding sequence was inserted. A fusion protein of the expected size was detected in a transformed *L. lactis* subsp. *lactis* strain by using Western blotting (immunoblotting).

During the last few years, lactococcal genetics has become an increasingly important field of study. Transformation and gene cloning systems have been developed that have been successfully used for the cloning and characterization of several homologous genes. Now that gene expression signals from *Lactococcus lactis* subsp. *cremoris* chromosomal DNA have been isolated and characterized in our laboratory (30, 31), the expression of a wide range of heterologous genes should become feasible. This might hold interesting possibilities for the future, especially since the lactococci are regarded as "GRAS" organisms (generally regarded as safe) (26) and therefore may be used for safe, large-scale production of commercially important proteins.

Few reports exist on the expression of heterologous genes in the lactic streptococci, except for a number of antibiotic resistance markers used in different cloning vehicles (6). As far as eucaryotic DNA sequences are involved, two studies report the expression of the (truncated) bovine prochymosine gene in *L. lactis* subsp. *lactis* (13; G. Simons and W. M. de Vos, Proc. 4th European Congr. Biotechnol., p. 458, 1987).

In this paper we report on the construction of a lactococcal expression vector and the expression of a fusion gene containing the eucaryotic hen egg white lysozyme (HEL)-coding sequence in *L. lactis* subsp. *lactis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1.

Escherichia coli and *Bacillus subtilis* were grown in TY broth (22) or on TY solidified with 1.5% agar. In *B. subtilis* protoplast transformation, DM3 was used as the plating medium (4). *L. lactis* was grown in glucose M17 broth (25) or on glucose M17 solidified with 1.5% agar. To osmotically stabilize electroporated cells, sucrose (0.3 M) was added to these media.

Kanamycin was routinely used at a concentration of 50 or 20 μg/ml for *E. coli* and *B. subtilis*, respectively, except for DM3 plates, in which 100 μg/ml was used. Erythromycin was used at 100 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*.

Isolation of plasmid DNA, restriction enzyme analysis, and gene cloning. Plasmid DNA was isolated essentially by the method of Birnboim and Doly (2). Restriction enzymes, Klenow enzyme, and T4 DNA ligase were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany) and used according to instructions of the supplier. The method of Mandel and Higa (17) was used to transform *E. coli*. Protoplasts of *B. subtilis* were prepared and transformed by using the procedure of Chang and Cohen (4). Plasmids were introduced into *L. lactis* subsp. *lactis* IL1403 by electroporation (29).

Gene product analysis. In vitro transcription and translation were performed by using a procaryotic DNA-directed translation kit (Amersham International, Amersham, England). Lysates of *L. lactis* for use in Western blot (immunoblot) analysis were prepared according to the following protocol. Strains were grown overnight in glucose M17 broth supplemented with 40 mM DL-threonine. Samples (10 ml) of this culture were centrifuged. The cell pellet was washed with 10 ml of STE (100 mM NaCl, 10 mM Tris hydrochloride, pH 7, 1 mM EDTA), suspended in 1 ml of STE, and transferred to an Eppendorf tube. Cells were spun down, and the pellet was suspended in 50 μl of a 10 mM Tris hydrochloride–1 mM EDTA solution (pH 7) containing mutanolysin (150 U/ml) and $MgCl_2$ (1 mM). After incubation at 37°C for 30 min, 1 volume of 2× loading buffer for polyacrylamide gel electrophoresis (23) was added, and the samples were heated to 100°C for 10 min. Cell debris was removed by centrifugation, and samples of the supernatant (typically 1/10 volume) were used for further analysis.

In vitro transcription-translation products as well as cell lysates were applied to 15% sodium dodecyl sulfate-polyacrylamide gels (16) and subjected to electrophoresis. In vitro transcription-translation products were subsequently visualized by autoradiography. The electrophoretically separated proteins from the cell lysates were electroblotted onto nitrocellulose, after which HEL sequences were detected by using a rabbit anti-HEL antiserum and peroxidase-conjugated swine antirabbit immunoglobulins (Dakopatts, Glostrup, Denmark).

To examine whether the lysozyme fusion protein was present in an active form in *L. lactis*, cell lysates were prepared by sonication at 0°C (six cycles of 20 s with intervals of 20 s; amplitude, 4 μm; in a Soniprep 150 [MSE

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant feature(s)	Source or reference
Bacteria		
E. coli		
BHB2600		8
C600		1
JM103		18
MC1000		3
B. subtilis PSL1		20
L. lactis subsp. *lactis* IL1403		5
Plasmids		
pE194	Em^r	9
pGK11	Cm^r	15
pGKV232	Cm^r Em^r	31
pGKV432	Cm^r Em^r; derivative of pGKV232	Laboratory collection
pJH1	Em^r Km^r Sm^r Tc^r	28
pKLZ61	Ap^r; plasmid containing HEL cDNA	10
pMG36	Km^r	This work
pMG36e	Em^r	This work
pMG36HEL	Km^r	This work
pMG36eHEL	Em^r	This work
pPJ1	Ap^r Km^r; pUC7 containing Km^r gene of *S. faecalis* plasmid pJH1	Laboratory collection
pUC7e	Ap^r Em^r; pUC7 containing Em^r gene of *Staphylococcus aureus* plasmid pE194	Laboratory collection
pUC18	Ap^r	32
pWV01	Cryptic plasmid of *L. lactis* subsp. *cremoris* Wg2	15

Ltd., Crawley, Sussex, United Kingdom]). After removal of cell debris by centrifugation, samples of the supernatant were added to a suspension of lyophilized *Micrococcus lysodeikticus* cells (Sigma Chemical Co., St. Louis, Mo.), and the ΔA_{450}, representing lysozyme activity, was measured according to instructions of the supplier.

RESULTS

Expression vector construction. The composition of the expression vector pMG36 is presented in Fig. 1. The vector is based on pWV01, originally obtained from *L. lactis* subsp. *cremoris* Wg2, which also replicates in *E. coli* and *B. subtilis*. The part of the vector containing the pWV01 origin of replication was obtained from pGK11 by digestion with *Sau*3A and filling in of the recessed ends with Klenow enzyme. The kanamycin resistance marker, originating from the *Streptococcus faecalis* plasmid pJH1, was obtained as a *Hin*dII fragment from plasmid pPJ1. These two fragments were ligated, and the ligation mixture was used to transform *E. coli* MC1000 with selection for kanamycin resistance. Two *Hin*dIII sites, present in the terminator region of the kanamycin resistance gene (28), were then removed by cutting with *Hin*dIII, filling in of the recessed ends with Klenow enzyme, religation, and transformation of *B. subtilis* protoplasts. This procedure should leave a terminator with a ΔG of −21.8 kcal/mol (~91.2 kJ/mol), as compared with −29.4 kcal/mol (~123.0 kJ/mol) for the original terminator (21, 27). The plasmid thus obtained contained a unique *Cla*I site which was used to introduce the pUC18 multiple cloning site and the presumed terminator sequence of the *L. lactis* subsp. *cremoris* Wg2 proteinase gene (14). The latter part was introduced because it is known that it is often beneficial to have a transcriptional terminator present in an expression vector downstream of the site in which to clone foreign genes (24). In fact, for technical reasons, two fragments of the proteinase gene and adjacent sequences were cloned: a *Hin*dIII-*Hae*II fragment (nucleotides 6532 to 6651 according to the previously reported sequence [14]) and a *Hae*II-*Fnu*D2 fragment (nucleotides 6970 to 7141) containing the terminator sequence. These and subsequent steps in the vector construction were performed by using *E. coli* as a host for cloning. Finally, part of the multiple cloning site was replaced by an *Eco*RI-*Sal*I fragment from pGKV432 containing a promoter, a ribosomal binding site, and the start of an open reading frame isolated from *L. lactis* subsp. *cremoris* Wg2 chromosomal DNA. This fragment is well characterized, and the complete nucleotide sequence is known (31). The resulting expression vector was designated pMG36. Note that by removing the *Hin*dIII sites in the terminator region of the kanamycin resistance gene, the *Hin*dIII site present in the multiple cloning site became unique in pMG36.

Cloning of HEL cDNA. A 400-base-pair cDNA fragment containing the mature HEL-coding sequence flanked by an *Eco*RI and a *Hin*dIII site was isolated from plasmid pKLZ61 (10) and inserted in pMG36. The complete DNA sequence of this fragment is known (10, 12, 19). This enabled us to align the open reading frames of pMG36 and the HEL gene. For this purpose, the *Eco*RI-recessed end upstream of the HEL gene was filled in with Klenow enzyme and ligated into the *Acc*I site of pMG36, which was also made blunt by using the Klenow enzyme. The *Hin*dIII sticky end, present immediately downstream of the stop codon of the lysozyme gene, was ligated into the unique *Hin*dIII site of pMG36. The correctness of the construction was checked by the reappearance of an *Eco*RI site at the point of the *Acc*I-*Eco*RI fusion. The resulting plasmid was designated pMG36HEL.

In vitro transcription and translation. pMG36HEL was subjected to in vitro transcription and translation (*E. coli* system) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). The results show the presence of a protein band of approximately 17 kilodaltons (kDa) which is absent in the control lane containing proteins encoded by pMG36. The size of this protein agrees well with that of the expected fusion protein, and therefore, we may conclude that pMG36HEL directed the synthesis of a fusion protein containing the HEL.

Transfer to *L. lactis* subsp. *lactis*. Kanamycin resistance, initially chosen for its ease of selection in *E. coli* and *B. subtilis*, has also been reported to be a useful selectable marker in *L. lactis* when neomycin instead of kanamycin is used as the selective antibiotic (7). However, in our hands, both neomycin and kanamycin selection were usually problematic in *L. lactis*, giving rise to high background levels of untransformed cells. Since selection for erythromycin resistance is much more efficient in *L. lactis*, the larger part of the kanamycin resistance gene was removed form pMG36HEL by digestion with *Eco*RV. This part was replaced by a 1-kilobase-pair fragment from pUC7e containing the erythromycin resistance gene from the *Staphylococcus aureus* plasmid pE194. The resulting plasmid, designated pMG36eHEL, was transferred to *L. lactis* subsp. *lactis*

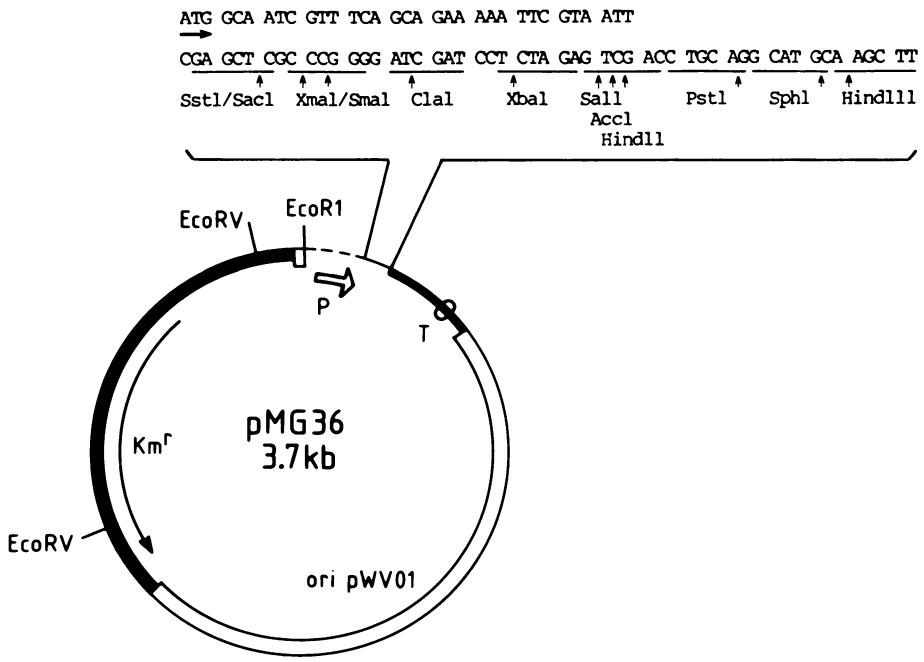

FIG. 1. Map of the expression vector pMG36 (size in kilobases [kb]) showing the start of the open reading frame and the multiple cloning site in expanded view. Symbols: ▭, pGK11-derived part containing the pWV01 origin of replication; ▬, pPJ1-derived part containing the kanamycin resistance gene; ——, sequence derived from the proteinase gene of *L. lactis* subsp. *cremoris* Wg2 containing a transcriptional terminator (T); - - - -, pGKV432-derived part (*Eco*RI to *Sal*I) containing a promoter (P), ribosomal binding site, and the start of an open reading frame (⇨).

IL1403 by electroporation with selection for erythromycin resistance. In the same way, the kanamycin resistance gene of pMG36 was replaced by the erythromycin resistance gene, resulting in the plasmid pMG36e. This plasmid was also transferred to *L. lactis* subsp. *lactis* IL1403.

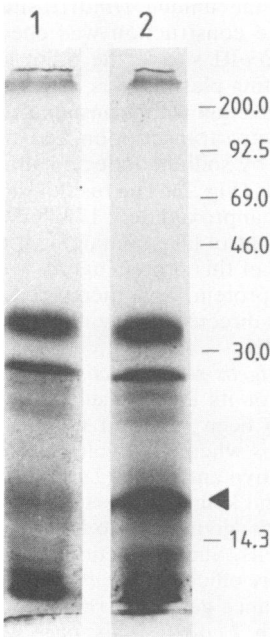

FIG. 2. In vitro transcription-translation products of pMG36 (lane 1) and pMG36HEL (lane 2). Protein molecular sizes are given in kilodaltons. The arrowhead marks the position of the 17-kDa fusion protein.

Expression of the lysozyme fusion gene in *L. lactis*. To examine whether lactococci are able to express the lysozyme fusion gene, lysates from *L. lactis*(pMG36e) and *L. lactis*(pMG36eHEL) were prepared as described in Materials and Methods. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently analyzed in a Western blot by using a rabbit anti-HEL antiserum (Fig. 3). The results show the presence of a 17-kDa protein in *L. lactis*(pMG36eHEL) which is absent in the strain containing pMG36e. This result strongly suggests that the HEL fusion gene is expressed in *L. lactis* subsp. *lactis* IL1403.

Besides this 17-kDa protein, several other protein bands are visible that show an identical pattern in both lysates and are thought to be due to aspecific staining reactions. The molecular weight marker used in this blot (Pre-stained SDS-

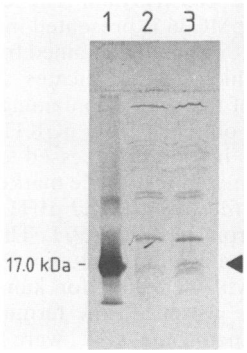

FIG. 3. Western blot of lysates of *L. lactis*(pMG36e) (lane 2) and *L. lactis*(pMG36eHEL) (lane 3). Lane 1: Molecular size standards. The arrowhead marks the position of the 17-kDa fusion protein.

PAGE MW Standards; Bio-Rad Laboratories, Richmond, Calif.) contains, among other proteins, the mature form of lysozyme. Because a dye is attached to the protein, it has an apparent molecular size of 17.0 kDa. This protein band becomes intensively stained in the Western blot, as would be expected.

With the observation that a 17-kDa protein was present in the *L. lactis*(pMG36eHEL) lysate, the question arose as to whether this gene fusion product was enzymatically active. To answer this question, lysates of the two plasmid-containing *L. lactis* strains were incubated with a suspension of *M. lysodeikticus* and the decrease in A_{450} was monitored. No difference was observed between the strain containing pMG36eHEL and the control strain containing pMG36e (results not shown). Therefore, it must be concluded either that the fusion protein was inactive or that it was produced in too low a quantity to be detected in this assay.

DISCUSSION

An expression vector for use in *L. lactis* was constructed which contains a multiple cloning site flanked by gene expression signals obtained from *L. lactis* subsp. *cremoris* Wg2. This plasmid, pMG36, contains the pWV01 origin of replication, which has been reported to be functional in a wide range of bacteria. These include *E. coli*, *B. subtilis*, lactococci (15), lactobacilli (B. M. Chassy, personal communication), *Streptococcus pyogenes*, and *Streptococcus sanguis* (J. Kok, personal communication). This feature facilitates the cloning of foreign genes, because *E. coli* and *B. subtilis* can be used as intermediate hosts for the construction of recombinant plasmids. Furthermore, it might extend the use of the vector to gene expression studies in other organisms that can recognize the *L. lactis* gene expression signals.

The multiple cloning site downstream of the translation initiation signals allows the insertion of a gene of interest in an appropriate reading frame, thereby creating an in-frame fusion with the start of the open reading frame present in the vector. The *Sal*I, *Acc*I, and *Hin*dII sites are especially useful in this respect. After these sites are filled in with Klenow enzyme, blunt-ended fragments can be inserted in three reading frames at positions within a 2-base-pair distance from each other.

Because the kanamycin resistance gene from *S. faecalis*, an easily selectable marker in *E. coli* and *B. subtilis*, turned out to be a problematic marker for selection in *L. lactis*, it was replaced by the erythromycin resistance gene from *Staphylococcus aureus*. This improved the selectability in *L. lactis* markedly. However, in other bacteria, the original vector, pMG36, with its kanamycin resistance marker, might turn out to be the plasmid of choice.

As a model for the expression of eucaryotic genes in *L. lactis*, the HEL gene was chosen. HEL is an extensively studied protein which has been used as a model in various fields of protein research (for a review, see reference 11). The gene has already been expressed in *E. coli*, in which the lysozyme was reported to accumulate as an inactive precipitate (10).

In the present study, the lysozyme was expressed from a fusion gene containing the mature-lysozyme-coding sequence preceded by the 5′ part of an unknown *L. lactis* subsp. *cremoris* Wg2 chromosomal gene and a short multiple-cloning-site-derived sequence. The fusion gene spans 462 base pairs as compared with 387 for the mature-lysozyme sequence, and the predicted molecular size of the fusion protein is 17.0 kDa, whereas HEL has a molecular size of 14.3 kDa. In lysates obtained from *L. lactis* (pMG36eHEL), a 17-kDa protein could be detected by using a rabbit anti-HEL antiserum, whereas this protein band was absent in lysates from *L. lactis*(pMG36e). In both lysates, several other protein bands were visible as a consequence of nonspecific staining reactions. The 17-kDa protein, however, is believed to represent the lysozyme fusion gene product. Arguments in favor of this explanation are: (i) the reproducible presence of this protein in lysates of *L. lactis*(pMG36eHEL) and its absence in control lysates; (ii) the size of the protein, which corresponds well with the size of the fusion protein expected (whereas out-of-frame fusions would give rise to prematurely terminated smaller proteins); (iii) the observation that the intensity of nonspecific staining when compared with the intensity of staining of the 17-kDa protein was variable in several repeats of the experiment.

In whole-cell lysates, no activity of the cloned lysozyme could be detected. This might be due to the presence of the N-terminal extension. Alternatively, the protein might be incorrectly folded, as has been reported to be the case in *E. coli* (10). The protein might also be produced in too low a quantity to be detected in the assay used.

The fact that no lysozyme activity could be detected is probably owing to an intrinsic property of this protein. We did show, however, that a protein of the expected size was produced in *L. lactis* and that the expression vector pMG36, or alternatively pMG36e, can be useful in gene expression studies in lactococci, as it might be in several other organisms.

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