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Stable *Escherichia coli-Clostridium acetobutylicum* Shuttle Vector for Secretion of Murine Tumor Necrosis Factor Alpha

J. THEYS,[1] S. NUYTS,[2] W. LANDUYT,[2] L. VAN MELLAERT,[1] C. DILLEN,[3] M. BÖHRINGER,[4] P. DÜRRE,[4] P. LAMBIN,[2] AND J. ANNÉ[1]*

Laboratories of Bacteriology[1] and Immunobiology,[3] Rega Institute, Katholieke Universiteit Leuven, and Experimental Radiobiology, University Hospital Gasthuisberg,[2] Leuven, Belgium, and Microbiology and Biotechnology, University Ulm, Ulm, Germany[4]

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Recombinant plasmids were constructed to secrete mouse tumor necrosis factor alpha (mTNF-α) from *Clostridium acetobutylicum*. The shuttle plasmids contained the clostridial endo-β1,4-glucanase (*eglA*) promoter and signal sequence that was fused in frame to the mTNF-α cDNA. The construction was first tested in *Escherichia coli* and then introduced in *C. acetobutylicum* DSM792 by electroporation. Controls confirmed the presence and stability of the recombinant plasmids in this organism. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an in vitro cytotoxic assay were used to monitor expression and secretion of mTNF-α during growth. Significant levels of biologically active mTNF-α were measured in both lysates and supernatants. The present report deals with investigations on the elaboration of a gene transfer system for cancer treatment using anaerobic bacteria.

The genus *Clostridium* comprises a heterogeneous group of rod-shaped, anaerobic, gram-positive, endospore-forming bacteria. Understanding the molecular biology of clostridia brings with it improved prospects for biotechnological exploitation. The development of suitable molecular tools, especially transformation procedures and specialized shuttle vectors, made it possible to introduce and express both homologous and heterologous genes in these microorganisms (20). Recently, attention has been paid to the use of nonpathogenic (24) or avirulent bacteria (18, 27) as delivery systems of therapeutic genes in anticancer therapy. These strategies have been shown to be safe in use. Colonization occurs selectively in tumors, not in normal tissues (12), and treatment can be stopped with suitable antibiotics (11).

Taken together, these technologies provide a new approach to use genetically engineered bacteria in the treatment of cancer. So far, constructs based on the pMTL500F shuttle vector with the *Escherichia coli* genes cytosine deaminase and nitroreductase have been developed (4, 17). The expression of the cloned genes has been evaluated in *Clostridium beijerinckii*, but the therapeutic value of these achievements has yet to be realized. In this context, the present investigations were aimed at the establishment of a recombinant *Clostridium acetobutylicum* that secretes mouse tumor necrosis factor alpha (mTNF-α). This therapeutic agent was selected because it is a cytokine with multiple antitumor effects (28). These include selective action on the neovasculature of tumors, stimulation of T-cell-mediated immunity, and direct cytotoxicity to tumor cells, mainly through induction of apoptosis (3, 15, 16, 40). Moreover, enhancement of the antiproliferative effect of tumor cells was demonstrated in vivo when TNF-α was combined with irradiation (6, 31). However, systemic toxicity hampers its straight use. Therefore, innovative strategies are required to increase the therapeutic efficacy of TNF-α treatment. One approach is to increase the effective concentration to which the tumor is exposed by using local treatment as proposed with the bacterial gene transfer system. Since we are specifically interested in studying the effects of introducing mTNF-α in the tumor microenvironment, it was necessary to construct a vector which after introduction in *Clostridium* provoked the secretion of the therapeutic agent.

This report describes the cloning of mTNF-α cDNA in a stable *E. coli-Clostridium* shuttle vector. As a proof of principle that mTNF-α can be produced by *C. acetobutylicum* DSM792, mTNF-α cDNA was placed under transcriptional control of the endo-β1,4-glucanase (*eglA*) promoter of *C. acetobutylicum* P262 and fused to the *eglA* signal sequence. Data on mTNF-α production by recombinant *C. acetobutylicum* DSM792 are presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *C. acetobutylicum* DSM792 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The strain was grown in 2× YT medium (26) or reinforced clostridial medium (Difco Laboratories, Detroit, Mich.) at 37°C in an anaerobic system (model 1024; Forma Scientific, Marietta, Ohio) with 90% N_2 and 10% H_2 with palladium as the catalyst. For analysis of mTNF-α production, *Clostridium* was cultivated in 5-ml aliquots of 2× YT medium after 1/10 inoculation with an overnight culture. The medium was buffered at pH 7.2 with filter-sterilized morpholinepropanesulfonic acid (MOPS) (Sigma) added at a final concentration of 50 mM.

For primary vector constructions, *E. coli* TG1 (29) was used. In vitro mutagenesis was carried out with *E. coli* JM109 (29) and *E. coli* BMH 71-18mut S (37). These strains were regularly grown in Luria-Bertani broth at 37°C (300 rpm). Prior to electroporation, plasmids were methylated in vivo in *E. coli* ER2275 (pAN1) (22). After isolation, plasmid DNA preparations were desalted by two spins in Microcon 100 microconcentrators (Amicon, Inc., Beverly, Mass.) as recommended by the manufacturer.

Media were supplemented, when applicable, with erythromycin (25 μg/ml), ampicillin (50 μg/ml), chloramphenicol (35 μg/ml), isopropyl-β-thiogalactopyranoside (IPTG; 50 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal; 40 μg/ml).

Shuttle vectors used in this study are listed in Table 1. Plasmid pHZ117, containing the *eglA* gene of *C. acetobutylicum* P262, was a gift from H. Zappe (39). The mTNF-α cDNA was available on plasmid pIG2mTNF (Innogenetics, Ghent, Belgium).

* Corresponding author. Mailing address: Laboratory of Bacteriology, Rega Instituut, K. U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Phone: 3216337371. Fax: 3216337340. E-mail: Jozef.Anne@rega.kuleuven.ac.be.

TABLE 1. Shuttle vectors used in this study

Plasmid	Size (kb)	Parent plasmid		Selectable marker[a]		Reference
		Gram +	Gram −	Gram +	Gram −	
pKNT19	4.9	pIM13	pUC19	EM (MLS^r)	AP	2
pIMP1	4.7	pIM13	pUC9	EM (MLS^r)	AP	21
pMTL500E	6.4	pAMβ1	pMTL20	EM	AP	23

[a] EM, erythromycin; AP, ampicillin, MLS^r, macrolide-lincosamide-streptogramin B resistance.

Transformation procedures and DNA manipulations. *E. coli* was transformed by using chemically competent cells obtained with the RbCl method. Transformation of *C. acetobutylicum* was carried out by electroporation as recently published (25). In brief, cells were grown in 50 ml of reinforced clostridial medium until mid-log phase, washed with ice-cold electrotransformation buffer (270 mM sucrose, 0.6 mM Na_2HPO_4, 4.4 mM NaH_2PO_4, 10 mM $MgCl_2$ [pH 6]), and finally resuspended in 2 ml of electrotransformation buffer without $MgCl_2$. A 0.6-ml sample of the cell suspension was transferred to a 0.4-cm electroporation cuvette containing 1 to 5 μl of methylated plasmid DNA (0.1 to 1.5 μg). After the pulse (1.8 kV, 600 Ω, 50 μF), cell suspensions were diluted with 1.4 ml of 2× YT medium and incubated for 4 h at 37°C, before the cells were spread on selective plates and incubated for 3 to 5 days at 37°C.

All general DNA manipulations in *E. coli* were carried out essentially as described by Sambrook et al. (29). Restriction endonucleases and DNA-modifying enzymes were purchased from Roche Diagnostics (Brussels, Belgium), GIBCO BRL (Gaithersburg, Md.), and Eurogentec (Seraing, Belgium) and used as indicated by the suppliers.

DNA plasmid isolation from *E. coli* was performed with the Wizard Plus SV miniprep kit (Promega Inc., Madison, Wis.). Plasmid DNA was isolated from *C. acetobutylicum* by the alkaline lysis procedure described by Nakotte et al. (25).

Mutations were carried out with the Altered Sites in vitro mutagenesis kit from Promega. Oligonucleotides for mutagenesis were purchased from Eurogentec or Amersham Pharmacia Biotech. Oligonucleotides used were 5′-GCTGAAGCT TCAACAACATC-3′ (for introduction of the *Hin*dIII site in *eglA*) and 5′-GAC TACTTGATCTTACGTAGATTTAAACCTCCTG-3′ (for introduction of the *Sna*BI site in mTNF-α cDNA). The DNA fragments containing the introduced mutations were subcloned in pUC19 to verify the mutations by DNA sequencing with an automated laser fluorescent ALF DNA sequencer (Amersham Pharmacia Biotech). Primers used for sequencing were the fluorolabeled M13 universal and reverse primer.

DNA probes for Southern blotting experiments were labeled with digoxigenin. Labeling efficiency was checked by using chemiluminescent detection as recommended by the manufacturer (Roche Diagnostics). Southern blotting was performed with an optimized hybridization protocol (9).

Vector construction. pHZ117 was digested with *Hin*dIII/*Sac*I. The resulting 1.2-kb *eglA* fragment was isolated and subcloned in pSelect. A *Hin*dIII site was introduced four codons upstream of the signal sequence. A *Sna*BI site was created at the first codon of the mTNF-α cDNA, available on the pIG2mTNF plasmid, resulting in pIG2mTNF*Sna*BI (13). A 0.5-kb *Eco*RI/*Hin*dIII fragment containing the *eglA* promoter, ribosome binding site, and signal sequence was next cloned in pBR322. Restriction digestion with *Hin*dII and *Hin*dIII, followed by Klenow polymerase treatment, resulted in a 0.9-kb blunt-ended fragment. This fragment was isolated and ligated in the pIG2mTNF*Sna*BI vector that was previously digested with *Sna*BI. The obtained construct was designated pIG2eglAmTNF. Subsequently, the eglAmTNF fragment was digested from the pIG2eglAmTNF plasmid by *Eco*RI and inserted into the *Eco*RI site of the different shuttle vectors (Fig. 1).

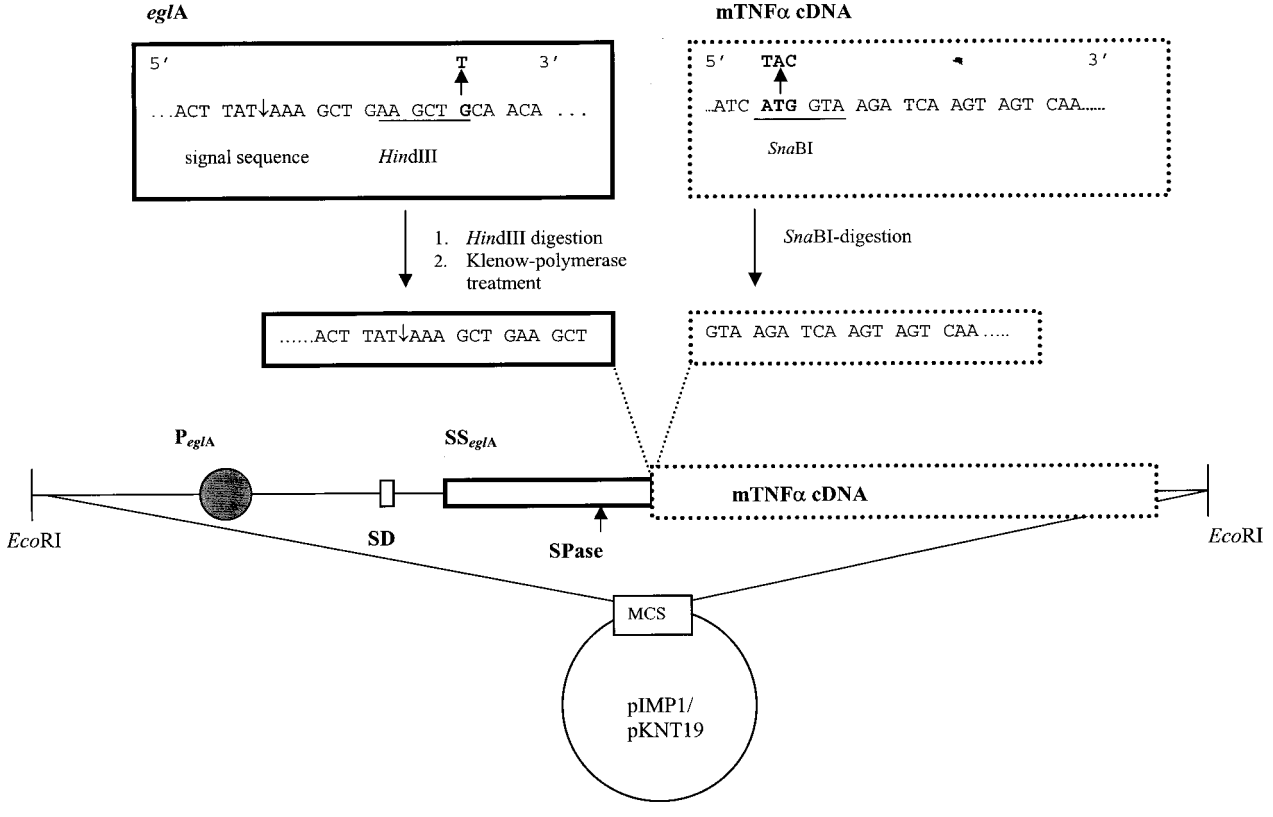

FIG. 1. Schematic representation of the construction of the pIMP1eglATNF and the pKNT19eglATNF shuttle vector and of the mutations introduced in the original *eglA* sequence and the mTNF-α cDNA. P_{eglA}, promoter region of *eglA*; SS_{eglA}, signal sequence of *eglA*; SD, Shine-Dalgarno sequence; SPase, signal peptidase cleavage site (indicated by ↑).

Determination of segregational plasmid stability. Five milliliters of fresh 2× YT medium containing erythromycin was inoculated with 0.5 ml of late-exponential-growth-phase cultures of recombinant *C. acetobutylicum* DSM792 strains. Cultures were subsequently subcultivated every day into 5 ml of fresh 2× YT medium without erythromycin pressure over a 6-day period. These cultures were diluted and subsequently plated onto both selective and nonselective 2× YT plates. The numbers of colonies on selective and nonselective growth media were compared to determine plasmid stability [expressed as a percentage; (number of Em^r colonies on selective plates)/(total number of colonies on nonselective plates) × 100]. Randomly isolated colonies from selective plates were examined for the presence of plasmid DNA.

Detection and quantification of mTNF-α. For mTNF-α quantification, cells were harvested at various time periods during growth. At each interval, 0.5 ml of cell suspension was pelleted by centrifugation (Sorvall MC; 12 V, 12,000 rpm, 2 min). The supernatant was removed and immediately stored at −80°C. The cell pellet was resuspended in 0.5 ml of 100 mM Tris-HCl (pH 7.4), and the cells were lysed by sonication with two pulses of 20 s. Cellular debris was thereafter pelleted by centrifugation (12,000 rpm, 2 min), and the cleared lysate was stored at −80°C. Subsequently, the amount of biologically active mTNF-α in lysates and supernatants was determined as described by Heremans et al. (8) by using recombinant mTNF-α (specific activity, 2.5×10^8 U mg^{-1}) produced in *E. coli* as the standard. Briefly, cytotoxicity of mTNF-α towards WEH164 clone 13 cells was spectrophotometrically evaluated via the in situ reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), measuring the percentage of dead cells.

Immunoblot analysis with polyclonal rabbit anti-mTNF-α antibodies was carried out by the method of Van Mellaert et al. (35).

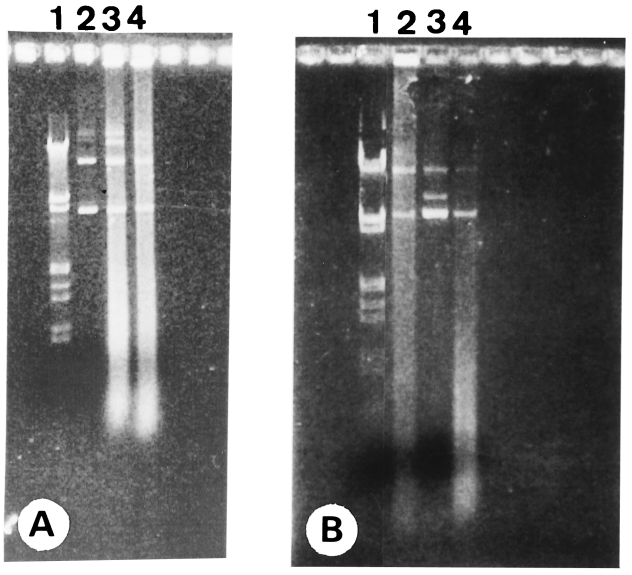

FIG. 2. Photograph of isolated plasmids pIMP1eglATNF and pKNT19eglATNF after gel electrophoresis on a 1% agarose gel stained with ethidium bromide. (A) Lanes: 1, λ DNA digested with *Eco*RI and *Hin*dIII; 2, pIMP1eglATNF isolated from recombinant *E. coli* TG1; 3 and 4, pIMP1eglATNF isolated from recombinant *C. acetobutylicum* DSM792. (B) Lanes: 1, λ DNA digested with *Eco*RI and *Hin*dIII; 3, pKNT19eglATNF isolated from recombinant *E. coli* TG1; 2 and 4, pKNT19eglATNF isolated from recombinant *C. acetobutylicum* DSM792.

RESULTS

Construction of shuttle vectors containing the mTNF-α cDNA and *Clostridium* transformation. The vectors used to construct recombinant plasmids containing the mTNF-α cDNA are listed in Table 1. To investigate the possibility of expressing and secreting mTNF-α in *Clostridium*, the promoter and signal sequence of the endo-β1,4-glucanase (*eglA*) gene of *C. acetobutylicum* P262 were chosen as a model. As outlined in Fig. 1, to obtain in-frame fusions between the *eglA* signal sequence and the mTNF-α coding sequence, restriction sites were created in the 3′ end of *eglA* as well as in the 5′ end of the mTNF-α cDNA. The introduction of the mutated restriction sites to fuse the regulatory sequence with the region at the start of the coding sequence ensured that the authenticity of the nucleotide sequence remained. Correct in-frame fusion was controlled by restriction digestion and by DNA sequence analysis. The *eglA* promoter is active in *E. coli*. As a consequence, it was possible to test lysates for the production of mTNF-α. Immunoblotting of the proteins of the cell lysates clearly demonstrated the presence of mTNF-α (data not shown).

Verification of the recombinant constructs in *C. acetobutylicum* DSM792. The recombinant constructs were methylated in vivo before they were introduced into *Clostridium* by electroporation. The obtained electroporation efficiency was $\sim 10^2$ transformants μg of DNA^{-1}. The recombinant plasmids were isolated from *C. acetobutylicum* by using a modified plasmid isolation protocol and visualized after electrophoresis on a 1% agarose gel and staining with ethidium bromide (Fig. 2). The pIMP1eglATNF and pKNT19eglATNF constructs were stably maintained in the clostridial host. This was confirmed by restriction digestion analysis and by transformation of *E. coli* with the isolated plasmid DNA. Southern blotting of isolated plasmid DNA either from *Clostridium* or from the transformed *E. coli* cultures with a digoxigenin-labeled specific mTNF-α probe resulted in the expected signal for the construct with pIMP1 and pKNT19 but not for the construct with pMTL500E (data not shown).

The segregational stability of both the pIMP1eglATNF and the pKNT19eglATNF construct was examined. Both plasmids were stably maintained following repeated culture transfer over a 6-day period in the absence of antibiotic pressure. The presence of plasmid DNA isolated from randomly selected colonies was confirmed by agarose gel electrophoresis (data not shown).

Immunoblot detection of mTNF-α in *C. acetobutylicum* (pIMP1eglATNF) and *C. acetobutylicum*(pKNT19eglATNF) cultures was carried out as described previously (35). Cultures of *C. acetobutylicum*(pIMP1), *C. acetobutylicum*(pKNT19), and plasmid-free *C. acetobutylicum* were taken as a control. Lysates and supernatants from overnight cultures were assessed for mTNF-α production. mTNF-α was detected in lysates of the recombinant cultures both as a preprotein (21 kDa) and as the mature, processed form (17 kDa). In the supernatant, however, only the mature form was present (data not shown). No mTNF-α was detected in lysates or supernatants of the *Clostridium* control cultures. These results clearly showed the functionality of the promoter and signal sequence preceding the mTNF-α cDNA.

Biological activity of produced mTNF. Samples of growing recombinant bacteria containing the pIMP1eglATNF or pKNT19eglATNF construct were taken at various stages of growth (Fig. 3A), and the biological activity of mTNF-α in lysates and supernatants was measured. Bacteria without a plasmid and bacteria with the pIMP1 or the pKNT19 vector alone were selected for controls. The activity was quantified by titration with the mTNF-α standard of known concentration (0.9 μg ml^{-1}). Several independent experiments were performed, and the results were normalized towards the titer of the first experiment so that the data could be pooled. The normalization also allowed the interexperiment comparison, showing confirmation for quantitative reproducibility.

The mTNF-α concentration in lysates and supernatants of recombinant clostridia containing the pIMP1eglATNF plasmid increased until the growing cells reached mid-log phase (optical density at 600 nm, ≅0.6). Thereafter, the amount of the mTNF-α present in the supernatant decreased below the de-

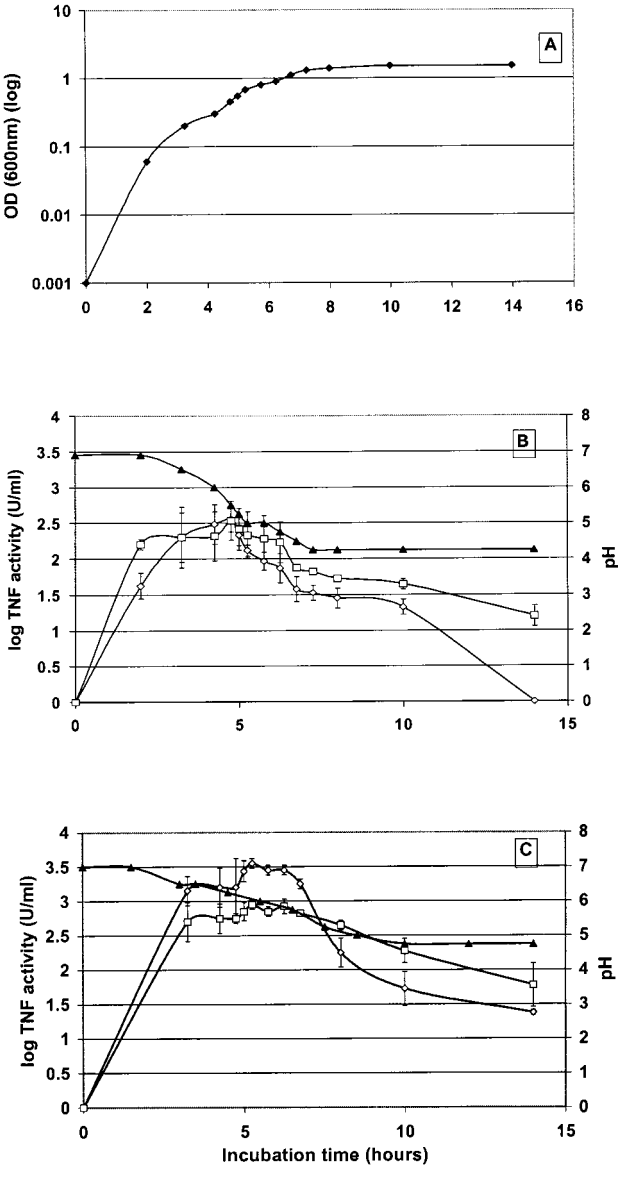

FIG. 3. Amount of biologically active mTNF-α in supernatant (◇) and lysates (□) of *C. acetobutylicum* DSM792 transformed with pIMP1eglATNF and evolution of pH (▲) in nonbuffered (B) and buffered (C) medium as a function of growth time (◆) (A). Error bars represent standard deviations.

tection limit (3.1 U ml^{-1}) after 12 h, whereas there was still biologically active mTNF-α in lysates for up to 20 h. Both in lysate and supernatant, a maximum of $\sim 10^3$ U of mTNF-α activity ml^{-1} was found (Fig. 3B). Very similar time-related mTNF-α activity measurements were obtained with the pKNT19eglATNF construct. In supernatants and lysates of cultures without the plasmid or with the plasmid not containing the mTNF-α gene, no mTNF-α activity was detected.

To test whether the decrease in activity was due to the action of proteases or due to the formation of acidic fermentation products from the recombinant clostridia, causing a pH decrease in the medium, MOPS (pH 7.2) was added to buffer the culture medium. This resulted in an elevated level of biologically active mTNF-α (Fig. 3C) in both lysates and supernatants (measured within a follow-up period of 24 h). From these data, we can conclude that the higher amount of active mTNF-α clearly corresponds with the slower decrease in pH in the buffered medium. This is completely in accordance with the literature describing the stability of TNF-α within a pH region of 10 to 5.5 (36).

DISCUSSION

The present experiments were carried out in the context of a tumor-specific gene transfer system using apathogenic clostridia (11, 12). We assessed whether the eukaryotic mTNF-α cDNA could be expressed in *Clostridium* and whether mTNF-α could subsequently be secreted. The mTNF-α production from the colonizing recombinant bacteria may lead to an improved in vivo antitumor response. This would likely occur in the absence of systemic toxicity because of the selective intratumoral deposition of the cytokine.

Therefore, *C. acetobutylicum* DSM792 was genetically engineered to produce mTNF-α. With these experiments, the presence of biologically active mTNF-α in culture supernatants and cell lysates of *C. acetobutylicum* DSM792(pIMP1eglATNF) and *C. acetobutylicum* DSM792(pKNT19eglATNF), containing the mTNF-α cDNA fused to the signal sequence of *eglA* and preceded by the *eglA* promoter, was clearly demonstrated both by Western blotting with mTNF-α antibodies and in a bioassay using WEH164 clone 13 cells. Notwithstanding that the mTNF-α gene was preceded by a signal sequence originating from *Clostridium*, not all of the mTNF-α expressed was also secreted. Secretion efficiency might possibly be improved by using a different signal peptide or by modifying, e.g., the number of positive charges in the signal peptide, as shown for other organisms (13).

Our experiments showed that the pIMP1 and pKNT19 derivatives were segregationally stable in *C. acetobutylicum* DSM792. Both constructs were structurally more stable than the construct with pMTL500E. It has been suggested that vectors based on the pAMβ1 replicon such as pMTL500E would be structurally more stable and thus more suitable for general use in *Clostridium* (38). This hypothesis relies on the absence of highly recombinogenic single-stranded DNA intermediates that would interfere with the structural stability of the plasmid. Our experiments, however, do not confirm this general hypothesis. Since other derivatives of pMTL500E have been shown in vitro to be stably maintained in *C. beijerinckii* (4, 17), these findings and our data considered together possibly reflect a strain- and/or sequence-specific character of vector stability.

Clostridium is characterized by a low G+C content with a strongly biased codon usage towards codons in which A and U predominate. However, since biologically active mTNF-α was detected in our experiments, the low G+C content of the host organism (28 to 29 mol%) seemingly has no implications for the expression of the cloned mTNF-α gene, at least in the strain used. Moreover, functional mTNF-α was secreted. Secretion was possible because of the presence of a clostridial signal sequence (39). To our knowledge, this is the first report describing the secretion of a eukaryotic protein from *Clostridium*.

Anaerobic bacteria selectively colonize the hypoxic-necrotic areas of solid tumors. This has been demonstrated with rodent tumor models (11, 19) and is documented for some cancer patients (10, 32). Since these hypoxic-necrotic regions are not present in normal tissues, this transfer system is very tumor specific (12). As a consequence, it should be possible to increase the local concentration of therapeutic agents by using genetically modified anaerobic bacteria expressing and secreting these compounds. The antitumor effectiveness will obviously depend on the stability of the recombinant constructs

and on the expression of the therapeutic genes in an in vivo tumor system. In vivo results described by Minton et al., using the EMT6 mouse tumor model, indicated the difficulty in obtaining sufficient amounts of therapeutic proteins (24). This might relate to the stability of the constructs and/or to the insufficient *Clostridium* colonization of the tumor. Using another tumor model and different therapeutic gene products, we aim to improve the therapeutic efficiency in vivo. Separately, the ameliorated tumor colonization of rat rhabdomyosarcomas using a vascular targeting compound has recently been demonstrated in our laboratories (14).

The clinical usefulness of systemically administered mTNF-α is limited due to hepatotoxicity and hypotension as major dose-limiting side effects (28, 30). The strategy introduced in the present investigations aims to restrict the antitumor potential of this cytokine solely in the tumor site, thereby bypassing the systemic toxicity.

TNF-α has been shown to be cytotoxic towards many transformed cell lines in vitro (5, 33). Antitumor activity against a variety of murine as well as human tumors has also been documented with in vivo investigations (1, 7, 34). Experiments are in progress to analyze in vivo the qualitative and quantitative effects of mTNF-α expression in rodent tumor models.

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